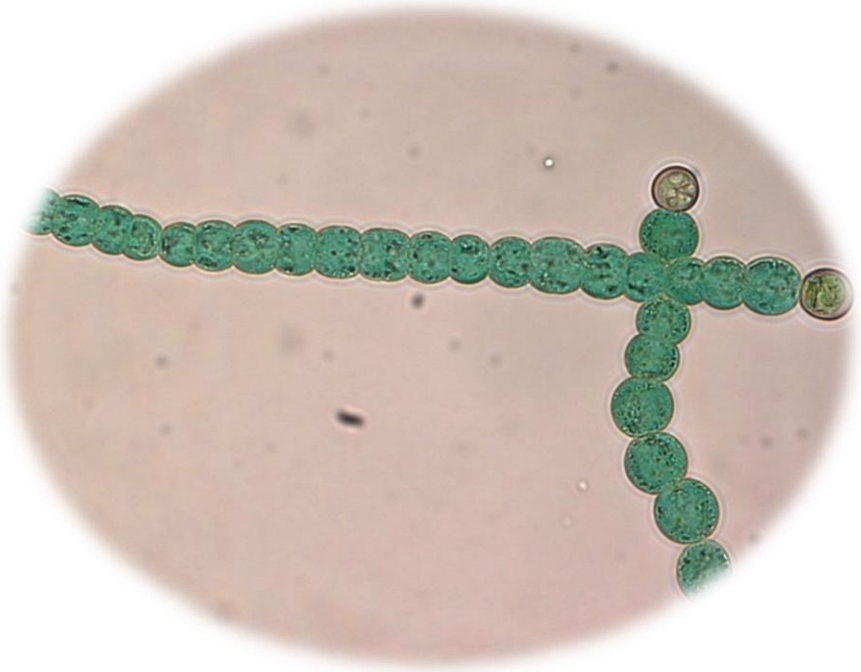
PHOSPHONATE DEGRADATION BY THE TOXIC BALTIC SEA CYANOBACTERIUM *NODULARIA SPUMIGENA*



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Tiivistelmä/Referat –**Abstract**

Inorganic phosphate (Pi) is the only readily utilizable form of phosphorus for toxic diazotrophic cyanobacterium *Nodularia spumigena (N. spumigena)*. Pi is one of the limiting nutrients in the Baltic Sea where surprisingly *N. spumigena* are highly abundant especially during the summer. This indicates that *N. spumigena* possibly has an alternative pathway to fulfill its phosphorus requirement. The Baltic Sea, like most aquatic environments, is enriched with organic phosphorus compounds among which phosphonates may constitute a significant fraction. Interestingly, the Baltic Sea *N. spumigena* strains UHCC 0039 and CCY9414 have been found to carry phosphonate degrading gene cluster (*phnC-M*) implying that these cyanobacteria could assimilate phosphonates as a phosphorus source. However, the significance of the presence of *phn* gene cluster in *N. spumigena* for phosphonate utilization has not been investigated in detail. Here, I aimed to understand how *N. spumigena* copes with Pi limitation and utilizes phosphonates in laboratory conditions using biochemical assays, PCR-based methods and bioinformatics tools. This would aid in finding a suitable marker for Pi deficiency in cyanobacterial blooms in the Baltic Sea.

In this study, bioinformatics and PCR screening showed that *phn* gene cluster was conserved in the Baltic Sea *N. spumigena* strains. The studied *N. spumigena* strains UHCC 0039 and UHCC 0060 were found to utilize naturally produced low molecular weight phosphonates, methylphosphonate (MPn), ethylphosphonate (EPn) and 2-aminoethylphosphonate (2APn). Among these phosphonates, MPn seemed to be the most preferred phosphorus source. Alkaline phosphatase activity, an indicator of Pi limitation, was found to be elevated in the media with Pi and 2APn questioning its suitability as a marker for phosphorus limitation. In addition, growth on MPn released methane indicating that massive blooms of *N. spumigena* might contribute to an elevated methane supersaturation in the Baltic Sea. Reverse transcriptase quantitative PCR (RT-qPCR) in *N. spumigena* strains did not show expected upregulation of high-affinity phosphate transporter *pstS* in Pi limitation. It demonstrated an induction of phosphonate transporter gene *phnD* in media lacking Pi and supplemented by 2APn. The phosphonate lyase gene *phnJ* was however, upregulated only in the presence of MPn suggesting that *phnJ* gene could be used as a marker for phosphonate bioavailability. The findings from this study suggest that the presence of *phn* gene cluster could provide *N. spumigena* a competitive advantage in Pi-limited cyanobacterial blooms in the Baltic Sea.

The molecular detection methods designed in this study thus could be used in future to monitor the expression of genes induced during Pi limitation and the presence of phosphonates, and the method could be further optimized for screening natural water samples.

Avainsanat – Nyckelord – Keywords
Cyanobacteria, inorganic phosphorus, Baltic Sea, *N. spumigena,* RT-qPCR

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TABLE OF CONTENTS

ABBREVIATIONS

2APn	2-Aminoethylphosphonate
arsB	Arsenite efflux protein gene
bp	Base pairs
Mbp	Mega base pairs
C	Carbon
cDNA	Complementary DNA
Chl *a*	Chlorophyll *a*
CO_2	Carbon dioxide
Ct	Threshold cycle in qPCR
DIP	Dissolved inorganic phosphate/orthophosphate
DNA	Deoxyribo nucleic acid
dsDNA	Double stranded DNA
DOP	Dissolved organic phosphorus
E.coli	*Escherichia coli*
EPn	Ethylphosphonate
GC	Gas Chromatography
gDNA	Genomic DNA
LMW	Low molecular weight
MPn	Methylphosphonate
N	Nitrogen
N_2	Nitrogen gas
Nodularia spumigena	*N. spumigena*
PCR	Polymerase chain reaction
pepM	Phosphoenolpyruvate phosphomutase gene
Pi	Inorganic phosphorus
Pit	Low affinity phosphate transporter
*phn*gene cluster	Phosphonate degrading gene cluster
phoA	Alkalinephosphatase
PSI	Photosystem I
PSII	Photosysten II
pstABCS	High-affinity phosphatetransporter
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
RT-qPCR	Reverse transcriptase quantitative PCR
SE	Standard error
sp.	Species
UHCC	University of Helsinki Cyanobacteria Culture Collection

LIST OF TABLES

LIST OF FIGURES

1. INTRODUCTION

Cyanobacteria are the ancient photosynthetic prokaryotes with an ability to fix atmospheric carbondioxide releasing oxygen. A majority of the cyanobacteria are diazotrophs i.e. fix atmospheric nitrogen (Whitton and Potts, 2012) and some can mineralize organic phosphorus by producing phosphatases (Singh et al., 2006). Thus, they play a crucial role in the primary production in the aquatic environment (Tyrell, 1999). Phosphorus is vital for cyanobacteria because it forms an integral component of the DNA, ATP and phospholipids. However, the most preferable form of phosphorus for cyanobacteria is inorganic phosphate (Pi), which is usually low or undetectable in the aquatic environment (Mcgrath et al, 2013; Villarreal et al., 2012). The Baltic Sea is one of the water ecosystems with low Pi concentration (0.1-0.01 µM) during summer (Nausch et al., 2004; Olofsson et al., 2016). However, massive accumulations of the toxic cyanobacterial blooms still occur making this brackish sea occasionally unsuitable environment for recreational purposes (Sivonen et al., 2007).

Bacteria and cyanobacteria have evolved several strategies to cope with the Pi limitation in the environment. The most studied response is the high-affinity phosphate transport system encoded by *pstABCS* operon, which is involved in sensing Pi level in the environment and thus allows effective Pi-uptake during Pi scarcity (Hirani et al., 2001). This operon is regulated via two-component signaling system PhoR/PhoB/PhoU (*pho* regulon), which is activated by autophosphorylation in Pi lacking environment. Another strategy to overcome Pi deficiency is the expression of gene encoding alkaline phosphatases that degrades phosphoesters (C-O-P), which occupy the major fraction (80-85%) of dissolved organic phosphorus (DOP) pool in aquatic system (Dyrhman et al., 2009). This gene is also located under *pho* regulon and the alkaline phosphatase activity is used generally as an indicator of Pi limitation (Van Wambeke et al., 2002). In addition, they have access to C-P bond containing phosphonates, which contributes ~10% to DOP pool (Young and Ingall, 2010). Phosphonates occur both naturally as side groups on glycoproteins or membrane phosphonolipids (methylphosphonate (MPn), ethylphopshonate (EPn), 2-aminoethylphsopnate (2APn) and anthropogenically (detergents, glyophosate and pesticides). The direct C-P bond is chemically and thermally inert and is resistant to chemical hydrolysis, thermal decomposition and photolysis (Metcalf and Wanner, 1993). Hence, they are widely used for industrial purposes in place of Pi and are being released in large quantities into the environment (Ternan et al., 1998). The abundant

presence of phosphonates has thus led to the evolution of several bacterial species with a capability to degrade these compounds to fulfill their requirements of carbon and phosphorus (Quinn et al., 2007; White and Metcalf, 2007; Villareal-Chiu et al., 2012).

One of the known enzymatic systems for the degradation of phosphonates is C-P lyase complex (Metcalf and Wanner, 1991). In *E. coli*, C-P lyase pathway is carried out by the phosphonate degrading operon (*phn* gene cluster) and is regulated by *pho* regulon. *phn* gene cluster consists of phosphonate transporter system encoded by *phnC-E* genes and C-P bond breaking complex encoded by *phnG-M* genes (Metcalf and Wanner, 1991; White and Metcalf, 2004). Studies have shown that the presence of *phn* gene cluster favors growth in an environment with phosphonates as a sole phosphorus source. This has been observed in different cyanobacterial strains such as *Trichodesmium* IMS101, *Synechococcus* sp., and *Anabaena cylindrica* PCC7122 (Dyhrman et al., 2006; Adams et al., 2008; Forlani et al., 2008). These cyanobacteria released greenhouse gas methane into the surrounding environment through degradation of MPn and thus contributed to methane supersaturation in the epipelagic regions of the marine ecosystems (Karl et al., 2008; Gomez-Garcia et al., 2011). Thus, the use of MPn releasing methane may shed light on the oceanic methane paradox, where surface water methane concentration is higher than the atmospheric equilibrium (Repeta et al., 2016).

N. spumigena is one of the dominant bloom forming diazotrophic cyanobacteria in the Baltic Sea that is widely known for its ability to produce hepatotoxin (Sivonen et al., 2007; Fewer et al., 2009). The ability of *N. spumigena* to proliferate successfully in the Pi limited Baltic Sea is intriguing. *N. spumigena* are known to produce alkaline phosphatases to utilize any available organic phosphorus compounds (Vahtera et al., 2007). In addition, the complete genome of the Baltic Sea *N. spumigena* CCY9414 showed the presence of *phn* gene cluster implying that phosphonates could be used as an alternative phosphorus source by this strain (Voβ et al., 2013). Furthermore, cyanobacteria capable of degrading MPn may play a crucial role in causing the increased methane concentration observed in the Baltic Sea surface water during the summer (Bange et al., 1994; 1998).

Studies on phosphonate pool of the Baltic Sea and their degradation by *N. spumigena* are lacking. Here, I studied how *N. spumigena* responds to the Pi scarcity and the presence of phosphonates. Both molecular and biochemical techniques were combined to find a suitable marker for Pi limitation and phosphonate bioavailability. Firstly, *phn* gene cluster was screened in sequenced *N. spumigena* strains to determine the order of *phn* gene cluster. *N. spumigena* specific primers were designed based on

the *phn* gene cluster containing cyanobacterial strains. PCR was performed to detect the *phn* gene cluster in *Nodularia* strains isolated from the Baltic Sea. Secondly, growth was followed in the Baltic Sea *N. spumigena* strains (UHCC 0039 and UHCC 0060) and *N. sphaerocarpa* UHCC 0038 (negative control, no *phn* gene cluster) in media with and without Pi and with phosphonates (MPn, EPn and 2APn), as the only source of phosphorus. Alkaline phosphatase activity was monitored to determine phosphorus status in the growth environment. In addition, the ability of *N. spumigena* to release methane from MPn was also studied. Furthermore, RT-qPCR was used to study the expression of *pstS* (phosphate binding gene), and the genes of *phn* gene cluster, *phnD* (phosphonate transporter) and *phnJ* (C-P bond cleaving) from cultures growing in a medium lacking Pi and containing MPn and 2APn.

2. LITERATURE REVIEW

2.1 Brief introduction to cyanobacteria

Cyanobacteria, also known as blue green algae are environmentally highly significant group of ancient photosynthetic gram-negative prokaryotes that produce oxygen as a side product of photosynthesis (Castenholz, 2001; Whitton and Potts, 2012). Photosynthesis occurs in specialized cellular structure called thylakoid membranes and it is carried out by two photosystems, photosytem I (PSI) and photosystem II (PSII). Phycobilisomes are the light harvesting complexes of PSI and it contains pigments such as phycocyanin and phycoerythrin which are responsible for their characteristics blue-green or red colors, respectively (MacColl, 1998). The pigment of PSII is chlorophyll *a* (chl *a*), which is the major light harvesting pigment (Castenholz, 2001). These pigments can be used as indicator of overall biomass or biomass of specific group of phytoplankton (Schagerl and Künzl 2007).

Fossils records estimate them to be ≥2100 million years old (Schopf, 2011). They are credited for the oxygenation of anaerobic Earth's atmosphere giving rise to multicellular eukaryotic life forms (Bhattacharya et al., 2003). Moreover, cyanobacteria are widely acknowledged for facilitating evolution of eukaryotic structures such as chloroplasts and plastids via endosymbiotic events with cyanobacteria where non-photosynthetic organisms engulfs a photosynthetic cell, which is then converted to plastid (Bhattacharya et al., 2003). Throughout their long evolutionary history, cyanobacteria have diversified into variety of species with different morphotypes and niche habitats. They are unicellular, filamentous or branched and can occur as single cells or in colonies (Castenholz, 2001). Cyanobacteria have inclusions in their cytoplasm such as glycogen, cyanophycin and polyphosphates granules for storage of carbon, nitrogen and phosphorus respectively (Castenholz, 2001). These storages can be used when there is nutrient limitation in the environment (Oliver and Ganff, 2000). Gas vesicles are present in many planktonic cyanobacteria that permit cell to regulate their buoyancy in the water column to obtain optimal light and nutrient conditions (Walsby, 1987). Some filamentous cyanobacteria are able to fix atmospheric nitrogen in special thick-walled cells called heterocycts (Haselkorn, 1978; Castenholz, 2001) and some can differentiate into akinetes (resting cell). Akinetes are not only capable of germination under beneficial conditions but also produced during adverse conditions such as nutrient deficiency and/or light scarcity and can tolerate drying, freezing and long-term

storage in anoxic sediments (Adams and Duggan; 1989; Castenholz, 2001). Likewise, hormogonia are often described as chains of 5-15 cells with diameter different from vegetative trichomes and they are usually motile (gliding) and often contain gas vacuoles when vegetative cells do not (Castenholz, 2001). Besides using water as an electron donor for photosynthesis, some strains can use hydrogen sulphide and release sulfur for anoxygenic photosynthesis. They can also form symbiotic associations with animals, plants and fungi (Carpenter and Foster, 2002). These aforementioned features have offered them ability to inhabit diverse array of habitats from extreme environments (hot-springs, glaciers, desert, UV-exposed surfaces, hypersaline lakes) to terrestrial, freshwater and marine environments across a range of trophic conditions (Tandeau de Marsac and Houmard, 1993; Mur et al., 1999).

Cyanobacteria are one of the major topics of interest for scientists because they cause mass occurrence called blooms in eutrophic water under favorable conditions (Conley et al, 2009; Paerl and Paul, 2012). On the one hand, these massive blooms are of great economic concern because they create physical disturbances by creating scums on the surface of water that affect tourism and recreational sectors (Sivonen et al., 2007; Hasselström, 2008). On the other hand, they have capability to produce wide variety of bioactive compounds, which may exhibit antifungal, antibacterial and anticancer properties (Singh et al., 2005). Some of them often produce toxins (neuro and hepatotoxins) posing a threat to animals and humans (Pearson et al., 2010; Chen et al., 2013). However, cyanobacterial blooms are also an asset to the ecosystem, as they contribute to the maintenance of homeostasis and nutrient stoichiometry in the aquatic ecosystem (Deutsch et al., 2007).

2.2 The Baltic Sea

The Baltic Sea is one of the largest nontidal, brackish water multi-basin ecosystems characterized a unique salinity gradient with freshwater in the coastal regions to almost marine water in the Kattegat. Salinity difference is caused as a result of large riverine freshwater inflow from drainage area and salty water through Danish Straits (Zillén et al., 2008). Due to restricted water exchange with the North Sea, the residence time of water in the Baltic is more than 30 years (Döös et al., 2004). There is perennial halocline at a depth of 60-80 m (Carstensen et al., 2014a). During long periods of stagnation, the deeper regions of the Baltic Sea remain hypoxic (oxygen deficient) (Carstensen et al., 2014a).

The Baltic environment is under profound anthropogenic influence of 85

million inhabitants in the surrounding areas (HELCOM 2009). Increased loads of phosphorus and nitrogen from different sources such as agricultural runoff, industrial wastes, and discharge from ships and sewage have elevated primary production and eutrophication in the Baltic Sea (HELCOM 2009, 2010). The high level of nitrogen elevates spring bloom of diatoms, while phosphorus play important role in the formation of diazotrophic summer cyanobacterial blooms (Vahtera et al., 2007). Following eutrophication, there is increased amount of algal decomposition and sedimentation of organic matter (Larsson et al, 1985). Degradation of the accumulated organic matter in the bottom sediments results in hypoxic conditions (Larsson et al, 1985) which, in turn cause internal phosphorus loading (Mortimer et al., 1941). The phosphorus fluxes from oxic conditions have been observed also in the Baltic Sea (Lukkari et al., 2009b). The increased phosphorus levels have thus further promoted diazotrophic harmful cyanobacterial blooms (Funkey et al., 2014). Phosphorus input to the Baltic Sea has been largely reduced (Gustafsson et al., 2012), resulting in improvement of coastal zones (Elmgren et al., 2015) nevertheless, uneven loading from point sources still occurs. Thus, phosphorus loads from coastal areas and the increased supply of decades old phosphorus from the bottom sediments under increasing hypoxic conditions (Carstensen et al., 2014a) make the Baltic Sea still susceptible to eutrophication and increased cyanobacterial blooms (Vahtera et al., 2007; Puttonen et al., 2014).

2.3 Cyanobacterial blooms in the Baltic Sea

The eutrophic Baltic Sea is a perfect environment where late summer cyanobacterial blooms are an annual phenomenon. The dominant blooming species during summer include diazotrophic cyanobacteria: *N. spumigena, Aphanizomenon* sp. and to lesser extent *Dolichospermum* sp. (former name *Anabaena* sp.) (Repka et al., 2004; Halinen et al., 2007; Koskenniemi et al., 2007). These cyanobacteria have been frequently found in the blooms in the Baltic Proper and the Gulf of Finland (Halinen et al; 2007; Suikkanen et al., 2007). The Baltic Sea strains of *N. spumigena* and *Dolichospermum* sp. may produce hepatotoxins known as nodularin and microcystins respectively (Sivonen et al., 1989; Halinen et al., 2007) whereas the toxicity of *Aphanizomenon* sp. has not been confirmed in the Baltic Sea (Repka et al., 2004; Wasmund et al., 2017). However, the freshwater species of *Aphanizomenon* are known to produce potent neurotoxins (Sivonen et al., 1989; Sivonen and Jones, 1999).

The Baltic Sea cyanobacterial blooms are considered to be centuries old (Bianchi et al., 2000), however, man-induced eutrophication has been suggested as a reason for increased bloom intensity since the beginning of 20th century (Finni et al., 2001). Several studies have shown that there are different factors controlling bloom formation. Generally, these include temperature, light, turbulence, upwelling and mixing of waters as physical factors, nutrients availability and salinity as chemical factors and grazing and lysis as biological factors (Paerl et al., 2001). It has also been suggested that low inorganic nitrogen to inorganic phosphorus ratio may enhance blue-green algae blooms over other eukaryotic algae (Paerl et al., 2001). In the Baltic Sea, warm water (>16-17 ˚C), calm weather, high irradiation and low inorganic nitrogen to inorganic phosphorus ratio (after spring blooms of diatoms) are found as contributing factors to success of diazotrophic cyanobacterial blooms (Wasmund, 1997).

2.4 *Nodularia spumigena*

N. spumigena belongs to genus *Nodularia* from order Nostocales (Castenholz, 2001). *N. spumigena* consists of filaments with varying length with or without heterocysts (Figure 1). The length and width of vegetative cells ranges from 2.7 µm to 6 µm and 5.7 µm to 12.3 µm respectively (Laamanen et al., 2001). Heterocysts are 4.2-7.6µm long and5.5-12.3µm wide (Lehtimäki et al., 2000; Laamanen et al., 2001).

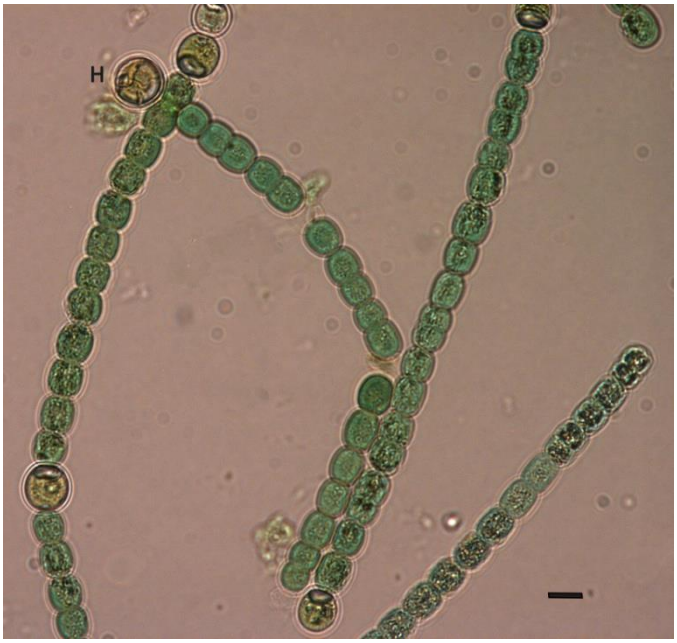

Figure 1 Photograph of a filament of the Baltic Sea *N. spumigena* UHCC 0039 used in this study showing thick-walled heterocyst (H) and vegetative cells. The 100x magnified picture is obtained from AxioCamHRc (ZEISS) connected to Axioskop 2 plus microscope. Scale bar= 6 µm.

N. spumigena have been shown to possess a distinct niche adaptation,

especially in brackish environments worldwide such as the Baltic Sea (Europe) (Voß et al., 2013), Peel-Harvey inlet (Western Australia) (Huber, 1984) or the Neuse River estuary (USA) (Paerl et al., 1991). In the Baltic Sea, the direct surface blooms primarily consist of *N. spumigena* and is more common in the central and southern part (Suikkanen et al., 2007; Wasmund et al., 2017). Despite the low phosphorus concentration (0.1-0.01 µM) (Nausch et al., 2004), *N. spumigena* occurs as one of the dominant bloom forming cyanobacteria in the Baltic Sea. The filamentous toxic *N. spumigena* are highly competitive towards other species because of their ability to fix nitrogen, control buoyancy, form aikinetes (Castenholz, 2001; Stal et al., 2003) and tolerate high radiation (Mohlin and Wulff, 2009). *N. spumigena* has been notorious for many cases of intoxication of animals in the coastal regions of the Baltic Sea (Edler et al., 1985; Simola et al., 2012).

2.5 Phosphorus as an element and a limiting nutrient for cyanobacteria

The chemical element phosphorus, with atomic number 15 is the eleventh most abundant element in the Earth's crust. Phosphorus can exist in different oxidation states: +5 as inorganic phosphate (PO_4^{3-}) or Pi, +3 (phosphite; PO^{3-}_3 and phosphonates, $C{-}PO(OR)_2$), +1 (hypophosphite; PO^{3-}_2) and -3 (phosphine gas; PH_3) (Karl, 2014). Phosphorus can exist in three bond forms: phosphomonoester (P-O-C) and phosphodiester (C-O-P-O-C), phosphonate (C-P) and polyphosphate (P-O-P-O-P) (Van Mooy et al., 2015).

Phosphorus is an essential macronutrient for all living organisms including cyanobacteria. It is present in the hydrophilic ends of lipids in cellular membranes that define membrane integrity. It forms the backbone of nucleic acids DNA and RNA via phosphodiester bonds in combination with sugars. The energy metabolism of cell is dependent upon the energy released from the hydrolysis of ATP and other nucleotides (McCleary, 2017). However, inorganic phosphate or orthophosphate ions $H_2PO_4^{2-}$, HPO_4^{2-}, and PO_4^{3-} (Pi) at oxidation state of +5 is the only directly utilizable form of phosphorus for cyanobacteria (Karl, 2014). These orthophosphate ions dominate the dissolved inorganic phosphorus (DIP) pool of aquatic environment and are one of the main growth-limiting nutrients for diazotrophic cyanobacteria (Sundareshwar et al., 2003).

2.6 Responses to Pi limitation in cyanobacteria

Cyanobacterial species have been found to harbor a genome of diverse sizes (~ 1.4-9.1 Mbp). Their diverse morphotypes and habitats with wide range of environmental fluctuations have evolved distinct characteristics in each of their genomes (Larsson et al., 2011). Cyanobacteria can quickly adapt to changes in environment by employing several strategies. Motility, photosynthetic adaptation, enzyme activities are short term strategies and induction or repression of either general or stress-specific genes, changes in cell metabolism, structure and morphology are long term strategies (Tandeau de Marsac and Houmard, 1993; Kopf et al., 2014; 2015).

Phosphorus depleted cyanobacteria on the one hand show different visible changes such as chlorosis (color change from blue-green to yellowish), smaller cells and sometimes foamy cultures (cell-lysis) as well as decrease in RNA content (Dignum et al., 2005). On the other hand, during Pi limitation, at the genetic level cyanobacteria are known to upregulate expression of series of genes of *pho* regulon (SphS/SphR, orthologous to PhoR/PhoB in *E. coli*) (Aiba et al., 1993; Hirani et al., 2001; Suzuki et al., 2004). *pho* regulon is a two-component regulatory system consisting of an inner-membrane sensory protein histidine kinase (PhoR) and a transcriptional response regulator in cytoplasm (PhoB) (Wanner, 1996). When there is a lack of Pi in the environment, PhoR is autophosphorylated and the activated PhoR phosphorylates PhoB, (Wanner, 1996) which bind to specific DNA sequences known as *pho* boxes (Makino et al., 1988) and either activates or deactivates the transcription of genes involved in P assimilation. This system often includes *phoU* (Santos-Beneit et al., 2008), which in environment with excess Pi, dephosphorylates PhoB by forming a repression complex (Wanner, 1996).

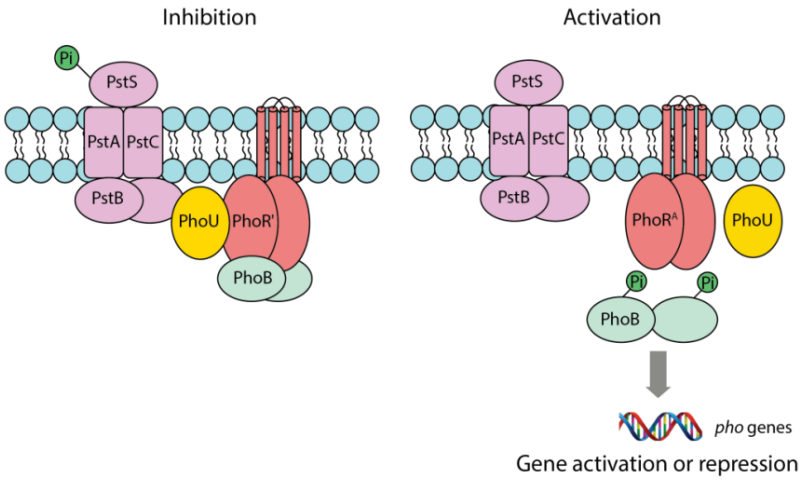

Figure 2 Schematic representations of *pho* regulon (modified from McGrath et al., 2013).

2.6.1 Activation of alkaline phosphatase activity

Alkaline phosphatases are the most common enzymes induced during Pi deficiency and are under control of *pho* regulon (Santos-Beneit et al., 2008). These enzymes liberate Pi from esters of organic phosphorus sources and measurement of alkaline phosphatase activity (APA) has been used generally as an indicator for Pi scarcity (Li et al., 1998; Nausch, 1998; Van Wambeke et al., 2002). Moreover, phosphoesters are widespread in various phosphorus biomolecules such as DNA, RNA, ATP and lipids. Phosphoesters occupy the major portion almost 80-85% of dissolved organic phosphorus (DOP) pool (Dyrhman et al., 2009) in aquatic ecosystem (Young and Ingall, 2010). Phosphoesterase such as alkaline phosphatases are located in the periplasmic space of cyanobacterial cell wall as soluble enzymes. Secretion of these enzymes is significant in fulfilling phosphorus demand in cyanobacteria (Bhaya et al., 2000). Thus, alkaline phosphatases are likely to be important in biogeochemical cycling of phosphorus as they hydrolyze Pi from different organic substrates (Kobayashi et al., 1984).

Alkaline phosphatases in prokaryotes have been divided into three families, PhoA, PhoD and PhoX. These families of alkaline phosphatases differ in their substrate specificities, metal requirements as cofactors and sub-cellular localization (Luo et al., 2009). In addition, PhoA and PhoD enzymes occur in the periplasm while PhoX are extracellular enzymes. In an *in-silico* study (Su et al., 2007), cyanobacteria was shown to contain putative *phoA* genes, which were not completely homologous to *E. coli phoA (*zinc dependent), suggesting other phosphatases might be present in marine systems. Further, study by Sebastian and Ammerman (2009) has shown that *phoX* (thought to be calcium dependent) is widely distributed in marine bacterioplankton including cyanobacteria such as *Trichodesmium*. However, *phoX* has been found to contain novel iron-calcium cofactor (Yong et al., 2014) indicating that iron, which is limiting in oceanic regions, could limit the hydrolysis of phosphomonoesters in marine cyanobacteria. Likewise, *phoD* was found to be widespread in both terrestrial and aquatic ecosystems near the ocean's surface. However, the relative abundance of these families of phosphatase in the deeper oceans is still undiscovered (Luo et al, 2009).

2.6.2 Increased Pi uptake

The most conserved proteins of the *pho* regulon in all bacteria include high affinity transporter system Pst (Santos-Beneit et al., 2008). The Pst system is additionally required for Pi signal transduction, as mutations in any genes of the *pst* operon result in

basic expression of *pho* regulon regardless of phosphate presence in the environment (Wanner, 1996; Hsieh and Wanner, 2010). In *E. coli*, the outer membrane contains PhoE protein, which, in case of Pi limiting conditions, helps in diffusion of phosphorus-containing compounds. These phosphorus-containing compounds when present in periplasm cannot pass through it due to their large size. The enzymes phosphatases hydrolyze these compounds forming Pi. The Pi then binds to the phosphorus binding protein (PstS) and is transported through the periplasmic membrane by the Pst system. Pst transporter system consists of PstA and PstC as integral membrane proteins and PstB as an ATP binding protein (Wanner, 1996; Hsieh and Wanner, 2010). In cyanobacteria, either whole *pstABCS* system is found to be highly expressed (Martiny et al., 2006) in phosphorus stress or it may be that only certain copies of *pstS* are upregulated and *pstABC* is constitutively expressed (Pitt et al., 2010). The expression of *pstS* gene thus has been the most studied genetic marker for Pi stress in bacteria including cyanobacteria (Su et al., 2007) and picocyanbacteria (Scanlan et al., 1993).

2.6.3 Phosphonate degrading gene cluster (*phn* gene cluster)

Phosphonates are organophosphorus compounds containing an inert, hydrolytically stable C-P bond with phosphorus at oxidation state +3. Phosphonates have been found to occupy 5-10% of DOP in marine environment (Young and Ingall, 2010). Phosphonates are both natural and human-made (Kononova and Nesmeyanova, 2002). In nature, phosphonates are synthesized in a reaction catalyzed by the enzyme phosphoenolpyruvate mutase (PepM) that involves the rearrangement of phosphoenol pyruvate (Villarreal-Chiu et al., 2012; Yu et al., 2013). The biogenic phosphonates includes 2-aminoethylphosphonate, methylphosphonate, ethylphosphonate and phosphoformic acid that are derived from degradation of phosphonolipids, glycolipids, glycoproteins and antibiotics (Kolowith et al., 2001; Dyrhman et al., 2006; White and Metcalf et al., 2007). The features provided by stable C-P bond i.e. resistance to chemical and enzymatic hydrolysis (Karl, 2014), has led to the production of various synthetic phosphonates in industrial applications, agriculture, pharmacy and households such as pesticides, detergent additives, antibiotics and flame retardants (Kononova and Nesmeyanova, 2002; Dyhrman et al., 2009; White and Metcalf, 2007, Metcalf et al., 2012).

It has been found that different heterotrophic bacteria and cyanobacteria can metabolize phosphonates via different pathways. These consist of C-P lyase pathway and substrate specific enzymes like phosphonoacetaldehyde hydrolase which, has been

described for hydrolysis of 2APn (White and Metcalf, 2007; McGrath et al., 2013). The former has been shown to be capable of hydrolyzing broad range of substrates and the most conserved pathway of phosphonate utilization (Kononova and Nesmeyanova, 2002). The microbial utilization of phosphonates via C-P lyase pathway consists of group of genes for degrading phosphonates called *phn* gene cluster. It is additionally believed to be located under *pho* regulon and has been found in many bacteria including cyanobacteria for transport and assimilation of phosphonates during Pi limitation (Cook et al., 1978; White and Metcalf, 2007). In *E. coli*, 10.9 kb *phn* operon consists of 14 genes (Figure 3).

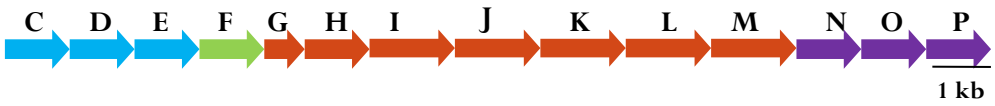

Figure 3 The organization of *phn* operon in *E. coli* K12 (Modified from Dyrhman et al., 2006).

Among 14 genes, *phnCDE* are thought to encode an ATP binding cassette transporter, C-P lyase multienzyme complex (*phn GHIJKLMNOP*) is required for hydrolysis of C-P bond (Metcalf and Wanner, 1993). PhnF and PhnNOP have been suggested as accessory proteins (Metcalf and Wanner, 1993; Hove-Jensen et al., 2010). In *Mycobacterium smegmatis*, the PhnF protein has been found to be a repressor of *phnC-E* (Gebhard and Cook, 2008).

Several studies have recently shown release of greenhouse gas methane from MPn degradation by C-P lyase pathway (White and Metcalf, 2007; Karl et al., 2008). Field experiments were conducted in mixed microbial communities in marine environment under Pi limitation (Karl et al., 2008). The study showed aerobic release of greenhouse gas methane from MPn that was likely to be mediated by C-P lyase pathway. Studies conducted later further supported results obtained from Karl et al. (2008) showing that MPn was present in marine archaeon *Nitrosopumilus maritimus* and that *pepM* gene, involved in biosynthesis of phosphonates was found in approximately 5% of sequenced bacterial genomes based on analysis of ocean metagenomic data (Yu et al., 2013) (Figure 4).

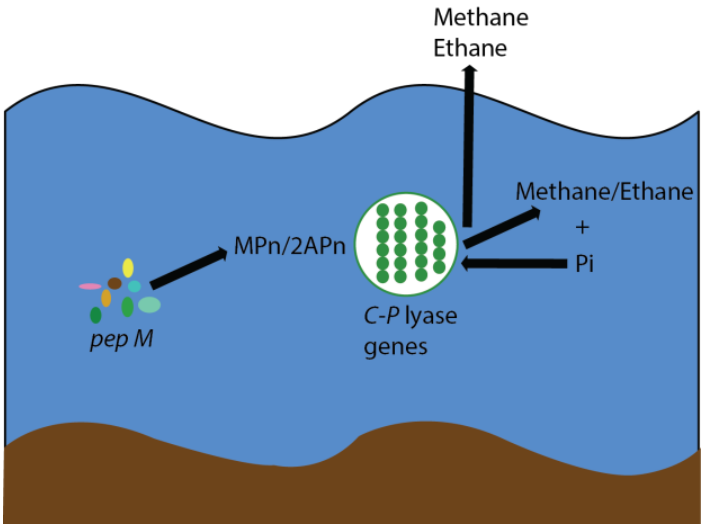

Figure 4 Phosphonate degradation catalyzed by C-P lyase gives alkanes and Pi. Organisms with *pepM* gene synthesize MPn and 2APn making it available to bacteria harboring *phn* gene cluster that utilize these phosphonates. Methane or ethane is released as an organic remnant and Pi is taken up bacteria (Modified from Repeta et al., 2016)

In addition, the anthropogenic production of phosphonates is a possible source of phosphorus to DOP pool (Quinn et al., 2007) indicating phosphonates can be an underappreciated pool of phosphorus for aquatic organisms. There are not enough studies on the detection of phosphonate in algae including cyanobacteria (Cade-Menun and Payatan, 2010). An only study by Dyrhman et al. (2009), has demonstrated the presence of about 10% phosphonates of total P in marine *Trichodesmium erythraeum* using ^{31}P NMR. Thus, phosphonates use by microorganisms as a source of phosphorus (Kononova and Nesmeyanova, 2002), as well as production of phosphonates by microorganisms may be an important component of phosphorus biogeochemical cycling and thus requires more attention (Benitez-Nelson et al., 2004).

2.7 Reverse Transcriptase quantitative PCR (RT-qPCR) in gene expression analysis

Polymerase chain reaction (PCR), a powerful tool in molecular biology, was developed by Kary Mullis and his associates in 1985 for which he was awarded Nobel Prize (Saiki et al., 1985; Kubista et al., 2006). It uses DNA polymerase enzyme in combination with two primers (forward and reverse) that are complementary to the target DNA sequence and deoxynucleotide triphosphates (dNTPs) to span a region of interest for amplification. PCR is a cyclic process that starts with heat denaturation of double stranded DNA (dsDNA), annealing of primers to single stranded DNA (ssDNA) and synthesis of DNA by DNA polymerase (Saiki et al., 1985). It creates multiple copies

(up to millions) of the target DNA called amplicons *in vitro*. The amplified products can then be detected by using gel or other fluorescent based techniques and can be further used for sequencing and cloning (Bevan et al., 1992).

Reverse transcriptase quantitative real time PCR (RT-qPCR) is a widely used PCR technique, which utilizes various fluorescent molecules to provide real time quantification of gene expression based on their intensities at specific biological conditions (Higuchi et al., 1993; Bustin et al., 2009). Unlike conventional PCR that records end-point amplification, qPCR monitors the amplification of nucleic acids in real time (Higuchi et al., 1993). Measurement of gene expression by RT-qPCR involves reverse transcription (RT), quantitative or real time PCR (qPCR) and quantification of PCR RT-qPCR data.

i. Reverse transcription (RT) is a process in which a complementary DNA (cDNA) is generated from RNA template by enzyme reverse transcriptase. cDNA is further used for PCR or quantitative PCR (qPCR) because RNA is less stable than DNA (Bustin, 2002). cDNA is also used for RNA-sequencing (RNA-seq) which is a popular technique for transcriptome analysis and gene expression estimation (Wang et al., 2009). Reverse transcriptase is a retroviral enzyme. It is used by retroviruses to incorporate its own coding RNA information into host DNA. It is mostly obtained from viruses such as Moloney murine leukemia virus or Avian myeloblastosis virus with increased thermostability and reduced endonuclease activity (Ståhlberg et al., 2003).

RT is the most susceptible step in terms of reproducibility (Bustin, 2002). RT reaction can occur with three different kinds of primer: random hexamers, gene specific primers and oligo-dT primers. Random hexamers are usually 6 base long primers and can bind to multiple places of single stranded RNA. So, there is high possibility of initiation of transcription. However, much of cDNA synthesized from total RNA is derived from ribosomal RNA or transfer RNA. This could cause problems if the mRNA of interest is present at very low levels, as priming of mRNA may not occur proportionately and its amplification may not be quantitative (Zhang and Byrne, 1999). Oligo-dT primers consist of 16-25 deoxythymidine residues and are more specific to mRNA and do not transcribe rRNA. They produce cDNA from 3′ end of polydenylated (polyA) of eukaryotic mRNA and cause 3′ bias of the synthesized products (Kubista et al., 2006). Gene specific primers bind to specific sequences and synthesize cDNA and are advantageous for their reduced amount of non-specific background cDNA. At the same time, these primer pairs require a new cDNA for each

gene included in the study (Kubista et al., 2006). Thus, all three primer types have both pros and cons.

ii. The major components of real time PCR detection system (such as CFX96 Touch^{TM}Real time PCR detection system, BIORAD) are: i) optical module to detect fluorescence in the reaction tubes during run and ii) thermocycler for PCR amplification. In addition, computer software is required for data collection and analysis of data (Valasek and Repa, 2005). During qPCR run, the analysis software will produce an amplification plot (Figure 5).

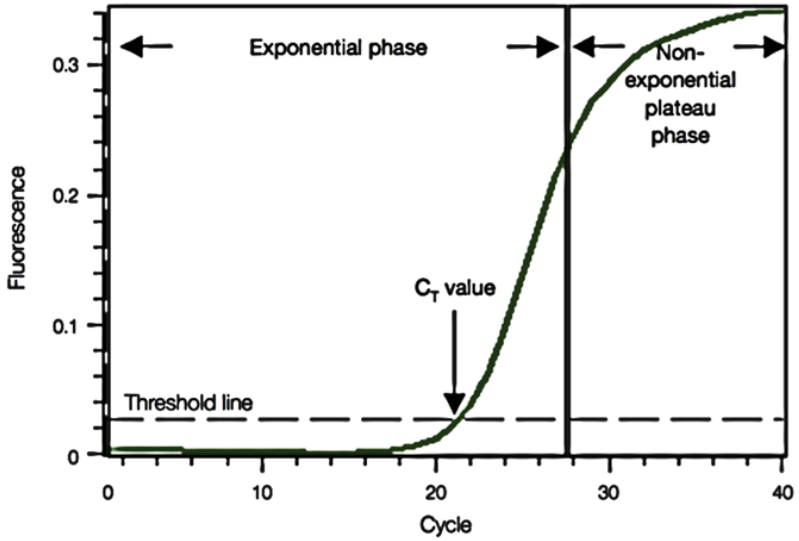

Figure 5 A sigmoidal shaped amplification plot showing fluorescence from amplification versus cycle number produced in linear scale (Bio-Rad, 2006). The fluorescence shown is subtracted from baseline.

The amplification plot (Figure 5) comprises of three phases. In the initial phase fluorescence measured is below the detection level. This level of fluorescence is the background of the reaction. The second phase also called an exponential phase is where fluorescence increases and the third phase called plateau phase where fluorescence is stable. Within exponential phase, it is possible to set a threshold value represented by a straight line in the plot indicating the area of exponential increase. The threshold differentiates background from the relevant amplification. The point at which the amplification curve and the threshold line intersect is called Cycle threshold (Ct), also known as Cq (quantification cycle). Ct is defined as the number of PCR cycles required to reach the level at which the fluorescent signal is first observed as statistically significant above background. Ct is inversely proportional to amount of template DNA and hence lesser number of cycles is needed to reach the threshold in the beginning of reaction (Valasek and Repa, 2005).

The qPCR uses two distinct types of fluorescent molecules: fluorescent dyes that bind to double stranded DNA (dsDNA) and dye-labelled sequence specific probes that bind to single stranded DNA (ssDNA). In this chapter, the qPCR with SYBR Green chemistry (Figure 6) will be discussed further.

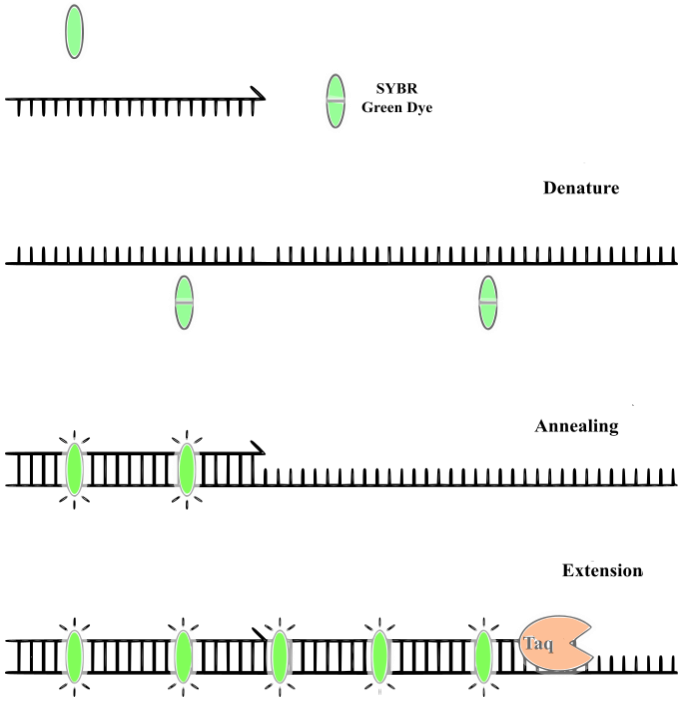

Figure 6 Graphical representation of how SYBR Green I dye results an increase in fluorescence in RT-qPCR. Free dye has low fluorescence as it does not bind to ssDNA or denatured DNA. As dsDNA is formed, SYBR Green I dye binds to it resulting in a dramatic increase in fluorescence (Adapted from Dorak, 2007).

SYBR Green I is a commonly used intercalating dye, which binds to double stranded DNA (dsDNA) and emits fluorescence. It absorbs light at 480 nM and emits light at 520 nM. Its fluorescence is 1000-folds greater when bound to DNA compared to its free state in solution (Valasek and Repa, 2005). The signal from dye is weak during initial cycles and cannot be differentiated from the background, but as the amount of dsDNA accumulates after each cycle, the fluorescence signal increases exponentially (Kubista et al., 2006). In case of dsDNA specific fluorescent dyes such as SYBR Green I, melting or dissociation curve analysis (Figure 7) can be used to discriminate sequences.

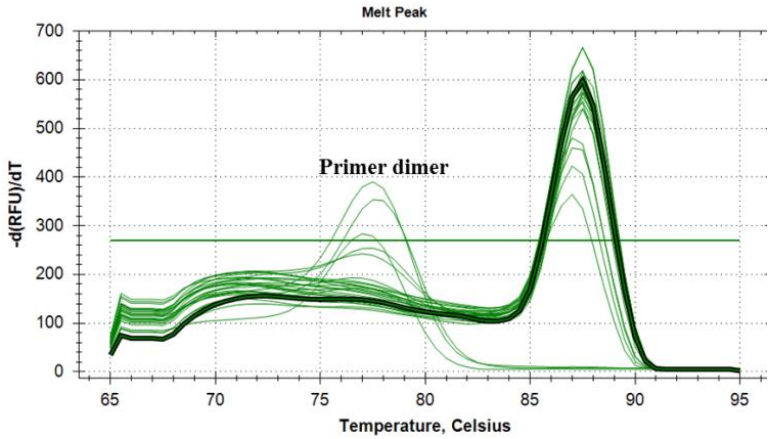

Figure 7 Melting curve showing primer dimer and product formation.

Melting curve at the end of qPCR run are generated that uses DNA binding dyes for detection when dsDNA is melted into single stranded DNA (ssDNA) with step-wise increase in temperature along with collection of fluorescence data at each temperature. Amplification products will melt at different temperatures depending on their base length and base composition thus leading different melt peak values. A single peak for each template containing reaction should be observed in ideal case whereas peak should not be detected in no template control (Bustin, 2005).

iii. There are two different methods of analyzing data from real time quantitative PCR experiments: (a) absolute quantification and (b) relative quantification. Absolute quantification is used when there is necessity to determine the absolute copy number of transcript of interest. It uses standard curve to relate PCR product to quantify the copy number of the target transcript (Livak and Schmittgen, 2001). Relative quantification is the method in which PCR signal of the target transcript in a treatment group is compared to that of untreated control group or reference sample also known as calibrator. Using reference genes or internal control genes is the most common method for normalization of gene expression. Internal reference genes are normally selected from housekeeping genes (Livak and Schmittgen, 2001). Housekeeping genes are those involved in basic cellular functioning and express abundant and constant expression despite varied experimental conditions (Eisenberg and Levanon, 2013). DNA replication is one of the most important functions of housekeeping genes. For instance, DNA gyrase (selected housekeeping gene in this project) is a heterotetrameric protein, which consists of two GyrA and GyrB subunits encoded by *gyrA* and *gyrB* genes respectively. These genes play crucial roles in DNA replication and segregation (Wang, 2002). There are several methods of relative quantification such as comparative

method, Pffal method (Bustin et al., 2002). Relative quantification by Comparative Ct ($2^{-\Delta\Delta Ct}$) method is explained in materials and methods (Section 4.4.5).

3. RESEARCH AIM

The abundance of toxic cyanobacteria *N. spumigena* in the Pi limited Baltic Sea may be attributed to its ability to adapt to an ecological niche by regulating different genes. The available genome data which, belongs to *N. spumigena* CCY9414 (5.46 Mb) isolated from the surface water near Bornholm in the southern Baltic (Voß et al., 2013) has revealed an extensive P acquisition system in the strain. The study has shown presence of both low affinity Pi transport system (Pit) and high affinity Pi transport system (PstABCS) implying that *N. spumigena* can assimilate Pi over wide range of concentrations (Voß et al., 2013). Genes encoding for phosphonate transport and lyase were also identified in *N. spumigena* CCY9414 suggesting its ability of alternative P assimilation system. However, the phosphorus acquisition mechanisms in the Baltic Sea *N. spumigena* have been poorly studied. Therefore, using biochemical and molecular based methods the distribution of *phn* gene cluster among *Nodularia* and Baltic Sea cyanobacteria was studied. Furthermore, the suitability of phosphonates as sole source of phosphorus was tested. This would help to obtain a reliable marker, which is indeed a necessity, for Pi limitation in the Baltic Sea cyanobacteria. The research aims were developed around the following questions:

1. Are *phn* gene cluster widespread in *Nodularia* strains?
2. What are the phosphonate sources that can be used by *N. spumigena* during Pi starvation?
3. Is methane gas a by-product of methylphosphonate degradation?
4. How were the genes of *phn* gene cluster expressed during Pi limitation and phosphonate bioavailability in *N. spumigena*?

4. MATERIALS AND METHOD

4.1 Outline of experiment

This research project can be divided into three tasks (Figure 8).

Task I: Screening of *phn* gene cluster

Bioinformatics screening of *phn* gene cluster from *N. spumigena* strains from GenBank. *In silico* design of primers was done using gene sequences from NCBI genome browser and in-house blast server containing newly sequenced and unpublished genomes of cyanobacteria. Gradient PCR and PCR were performed to check the specificity of the primers. PCR screening for *phnJ* and *phnD* genes in different *Nodularia* strains of University of Helsinki Culture Collection (UHCC).

Task II: Response to Pi limitation and phosphonates bioavailability

1. Growth experiment was set up with two Baltic Sea strains *N. spumigena* UHCC 0039 and 0060 and *N. sphaerocarpa* UHCC 0038. Chlorophyll *a* concentration was measured by spectrophotometer to estimate the cyanobacterial biomass.
2. Alkaline phosphatase activity (APA) assay was performed to determine the phosphorus status in growing medium by fluorometry.
3. Methane gas liberated from methylphosphonate was measured using gas chromatography.

Task III: Rt-qPCR assay design to study gene expression during Pi limitation

4. RNA extraction followed by cDNA synthesis and optimization of RT-qPCR were done.
5. The expression of two genes of *phn* gene cluster phosphonate transporter (*phnD)* and phosphonate lyase (*phnJ*) and high-affinity phosphate transporter (*pstS*) genes of *N. spumigena* UHCC 0039 and 0060 were analyzed by RT-qPCR. The *phnD* and *phnJ* gene expression was studied to gain insight of the phosphonates transportation and utilization. *pstS* was selected as it is the common genetic marker Pi starvation.

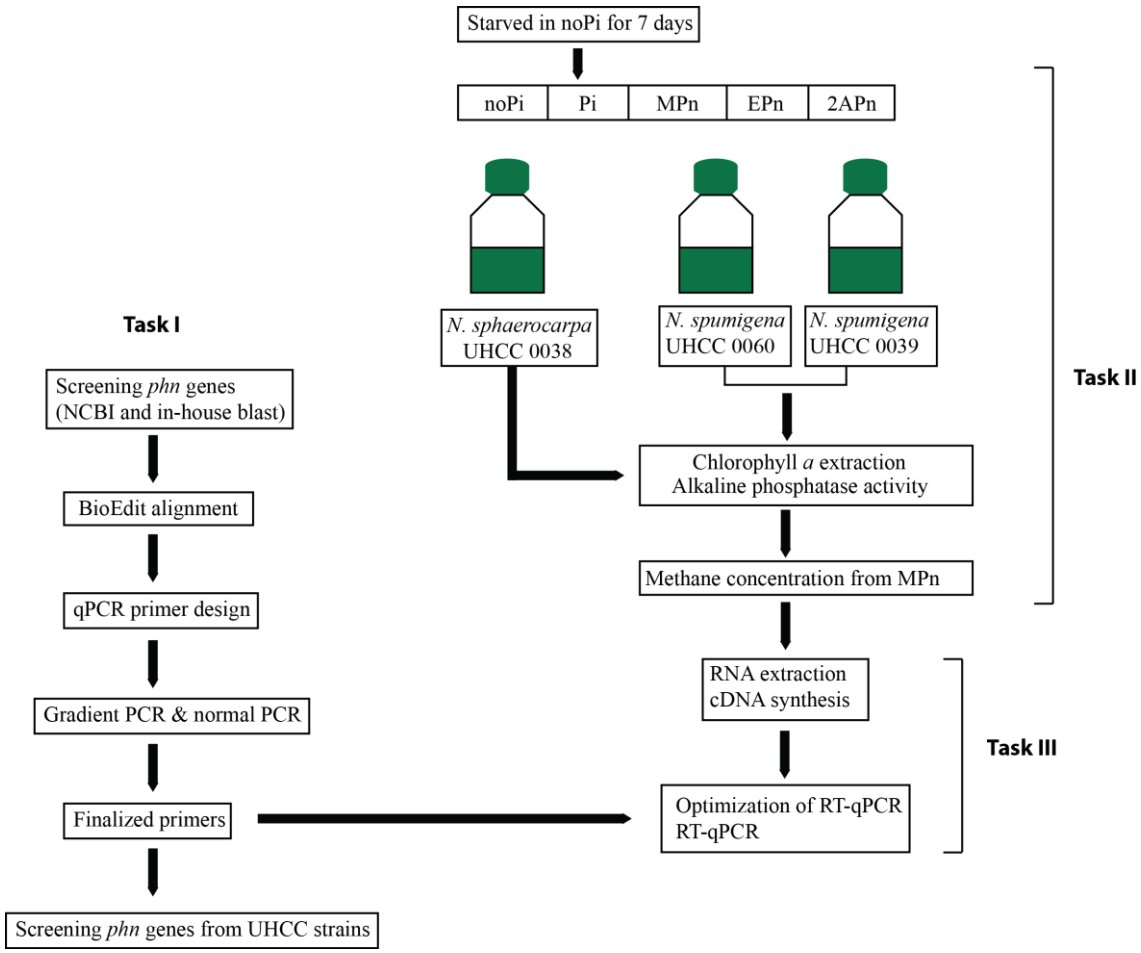

Figure 8 Testing the hypothesis that *N. spumigena* can utilize phosphonates as a source of phosphate

4.2 Task I: Screening of *phn* gene cluster

4.2.1 Bioinformatics screening of *phn* gene cluster

The phosphonate lyase proteins were identified from genomes of cyanobacteria available in National Center for Biotechnological Information (NCBI) GeneBank Database (http://www.ncbi.nlm.nih.gov). For this, the PhnJ sequences from *Escherichia coli* (*E.coli*) K12 and *N. spumigena* UHCC 0039(NCBI accession number, PRJNA352241), were used as queries in BlastP analysis. Among the cyanobacterial genomes encoding *phnJ* genes, the genomes of *Nodularia* strains *N. spumigena* CCY9414 (PRJNA13447) and *N. spumigena* CENA596 (PRJNA315832) were downloaded from the database to determine the order of *phn* gene cluster. Artemis genome browser was used to determine the gene order of *phn* gene cluster (Rutherford et al., 2000).

4.2.2 Primer design

In this study, primer pairs were designed for total four genes *phnJ*, *phnD* and *pstS* as target genes and housekeeping gene DNA gyrase subunit B (*gyrB*) for PCR based studies (Table 2). The primer design was based on alignment of sequences of *phn* gene cluster containing strains *N. spumigena* UHCC 0039 (NCBI accession number, PRJNA352241), *N. spumigena* CCY9414 (NCBI accession number, PRJNA13447) and *N. spumigena* CENA596 (NCBI accession number, PRJNA315832). The sequences for additional *Nodularia* strains and other cyanobacteria were downloaded from in-house cyanoblast and NCBI respectively to ensure that the primers designed would be specific for *Nodularia*. The information about strains used for primer design is given in Table 1. The sequences were aligned with ClustalW Multiple alignment in BioEdit program (Hall, 1999).

Table 1 Cyanobacterial strains used during primer design

Strains from GenBank	NCBI Accession number
Anabaena cylindrical PCC 7122	PRJNA43355
Cyanothece sp. PCC8801	PRJNA18951
Leptolyngbya sp. Heron Island J	PRJNA215004
Myxosarcina sp. GI1	PRJNA259928
*Nodularia spumigena*CCY 9414	PRJNA185469
Nodularia spumigena UHCC 0039	PRJNA352241
Nodularia spumigena CENA596	PRJNA315832
Nostoc PCC 7120	PRJNA244
Rubidibacter lacunae KORDI 51-2	PRJNA196392
Synechococcus sp. JA-2-3B'a(2-13)	PRJNA16252
Trichodesmium erythraeum IMS101	PRJNA318
Strains from in-house blast server	
Nodularia sp.UHCC 0040	
Nodulariaspumigena UHCC0060	
Nodularia sp. Sri	
Nodularia sp. CH309	

Since primers will be used for downstream RT-qPCR applications, primers were designed keeping into account the required criteria for successful qPCR. For instance, the qPCR amplicon size should be ideally between 80 and 150 bp but product size 80-200 bp is also acceptable. The optimal primer length should be 18-24 bp and melting temperature (T_m) should be between 59 and 68°C with maximum difference of 1-2 °C. The optimum annealing temperatures are generally 59 °C or 60 °C. It is also very necessary to prevent mispriming by avoiding long repeats and runs of nucleotides.

It is ideal to have 1 or 2 GC clamps that increase specificity by allowing primer to bind strongly to the template strand (Thornton and Basu, 2011).

Table 2 Primer sequences designed in this study. The bases covered by amplified regions of each gene in *N. spumigena* UHCC 0039 are presented.

Target gene Annotation	Bases position product size	Primer	Sequence 5'->3'	GC%	Melting temperature (℃)
phnJ	403-618	phnJF	TTCTAGGGCGTGCATTTTGC	50	58
C-P bond lyase	216 bp	phnJR	ACCAACGCCGTGAATATTCG	50	58
phnD	443-669	phnDF	GGTGCCTGCGGATTCTGACA	60	63
Phosphonate transporter	225 bp	phnDR	TAACATCGCCGCGTCATGAG	65	60
pstS	265-383	pstSF	GTTGCAGCCAATGGCACT	56	56
Phosphate binding	119 bp	pstSR	CTGACTTGTGCCAAACC	53	54
gyrB	1535-1723	gyrBF	CGCATATTCGCACACTGTTG	50	58
Gyrase subunit B	189 bp	gyrBR	TGTTGTAGTTGGCGTTGCTG	50	58

The designed primers were additionally analyzed in OligoCalc-progran to check GC%, predicted primer melting temperature (T_m) and self-complementarity of each primer (Kibbe, 2007). The specificity of primers for *Nodularia* was checked through BLASTn search (Altschul et al., 1990). Primers were ordered from Oligomer (Germany). All the primer pairs designed in this study were evaluated with gradient PCR and those producing strong single band of the expected size corresponding to the gene of interest were chosen for conventional PCR to test specificity and then for RT-qPCR for gene expression studies.

4.2.3 DNA extraction

Isolation of genomic DNA was done using the E.Z.N.A. SP Plant DNA kit (Omega Bio-Tek Georgia, USA) according to manufacturer's instructions with minor modifications. Cyanobacterial cells growing in approximately 40 mL of medium were collected by centrifugation at 7000 g for 7 min at 20 °C. After centrifugation, the pellets were mixed with 0.5 g of two different sizes of glass beads, 425-600 µM and 710-1180 µM (Sigma Aldrich, St. Louis, USA). To remove RNA, 5 µL of RNase A was added. Cells were broken by mechanical disruption using FastPrepTM-24 homogenizer (MP Biomedicals) at 6.5m/s for 20 s. The cells were then incubated at 65 °C 10 min. DNA concentration was measured using Nanodrop ND-1000 -spectrophotometer and stored at -20°C.

4.2.4 Gradient PCR

Gradient PCR was performed to find the best annealing temperature and check the specificity of the designed primers. The gradient PCR reaction was prepared using 50 µM of forward and reverse primer, 20 ng of template DNA of *N. spumigena* UHCC

0060, 0.4 µL of 10 mM dNTP (Thermo Scientific), 0.2 µL of Dynazyme DNA polymerase (Thermo Scientific, LOT: 00309224), 1 x Dynazyme buffer and sterile water to make the final volume 20 µL. For the primers designed in this study, annealing temperatures of 50, 50.8, 52.1, 53.9, 56.4, 58.3, 59.5, and 60 °C were included in the gradient. The gradient PCR protocol was as follows: initial denaturation of 95 °C for 3 min, 30 cycles of 95 °C for 30 s, the gradients of annealing temperatures for 30 s, 72 °C for 1 min and a final extension at 72 °C for 10 min.

4.2.5 Testing the specificity of primers by PCR

Different *N. spumigena* strains and *Dolichospermum* sp. strain were used during conventional PCR to check the specificity of designed primers (Table 3). The strains of cyanobacteria used during conventional PCR amplification of *phnJ* and *gyrB* were *N. spumigena* UHCC 0060, *N. spumigena* UHCC 0039, *N. sphaerocarpa* UHCC 0038 and *Nodularia* sp. UHCC 0040. For the PCR amplification of *phnD* and *pstS*, *N. spumigena* UHCC 0060, *Dolichospermum* sp. UHCC 0259 and *N. spumigena* UHCC 0042 were used. More information about strains is given in Table 3.

Table 3 Cyanobacterial strains from the Baltic Sea used for testing specificity of designed primers

Strain	**Former strain name**	**Year of isolation**	**Reference**
N. spumigena UHCC 0039	*N. spumigena* AV1	1987	1
N. spumigena UHCC 0042	*N. spumigena* AV33	1987	1
N. spumigena UHCC 0060	*N. spumigena* HEM	1987	1
N. sphaerocarpa UHCC 0038	*N. sphaerocarpa* HKVV	1986	2
Nodularia sp.UHCC 0040	*Nodularia* sp. AV2	1987	3
Dolichospermum sp.UHCC 0259	*Anabaena* sp. 259	2004	4

1: Sivonen et al., 1989; 2: Fewer et al., 2009; 3: UHCC; 4: Halinen et al., 2008

PCR reaction mix consisted of 2 µL Dynazyme II buffer (ThermoScientific), 0.4 µL of 10 mM dNTP (Thermoscientific), 0.3 µL of forward and reverse primer (Table 2), 1 µL of 20 ng template DNA and 0.2 µL of 0.4 U Dynazyme II (Thermoscientific) and autoclaved MilliQ water was added to make a final volume of 20 µL. Autoclaved MilliQ water was used as negative control. The PCR cycling parameters were as follows: initial denaturation of 95 °C for 3 min, 30 cycles of 95 °C for 30 sec, annealing temperature 60.5 °C for 30 sec, 72 °C for 1 min and a final extension at 72°C for 10 min.

4.2.6 PCR screening of *phnD* and *phnJ* genes from cyanobacterial strains from UHCC

phnJ and *phnD* genes were further screened using conventional PCR from strains belonging to University of Helsinki Culture Collection (HAMBI). All the strains were isolated from the Baltic Sea. More information can be found in Table 4. PCR amplification was performed as explained above (Section 4.2.5). A total of 13 cyanobacterial strains (Table 4) were screened for presence of *phnD* and *phnJ* genes. The strains from genus *Nodularia* included seven *N. spumigena* strains, two *N. sphaerocarpa* strains and four other *Nodularia* sp. (species unidentified). The genes were also screened from *Aphanizomenon flos-aquae* UHCC 0183. Here, *N. spumigena* UHCC 0060 was used as positive control and *N. sphaerocarpa* UHCC 0038 as negative control.

Table 4 Cyanobacterial strains isolated from the Baltic Sea used for *phnD* and *phnJ* screening

Strain	**Former strain name**	**Year of isolation**	**Reference**
N. spumigena UHCC 0060	*N. spumigena* HEM	1987	1
N. spumigena UHCC 0063	*N. spumigena* AV63	1987	1
N. spumigena UHCC 0184	*N. spumigena* TR183	1993	2
N. spumigena UHCC 0185	*N. spumigena* GR7a	1992	3
N. spumigena UHCC 0138	*N. spumigena* F81	1987	1
N. spumigena UHCC 0069	*N. spumigena* AN13c	Unknown	4
N. spumigena UHCC 0083	*N. spumigena* FL2a	1994	3
N. sphaerocarpa UHCC 0052	*N. sphaerocarpa* UP16f	1994	2
N. sphaerocarpa UHCC 0038	*N. sphaerocarpa* HKVV	1986	2
Nodularia sp. UHCC 0099	*Nodularia* sp. TRO12C	1994	3
Nodularia sp. UHCC 0075	*Nodularia* sp. WP2e	Unknown	3
Nodularia sp. UHCC 0158	*Nodularia* sp. GR7c	1992	3
Aphanizomenon flos-aquae UHCC 0183	*Aphanizomenon* TR183	1993	5

1: Sivonen et al., 1989; 2: Fewer et al., 2009; 3: UHCC; 4: Allahyerdiyeva et al., 2010, 5: Lyra et al., 2001

4.2.7 Gel electrophoresis to visualize PCR products

Gradient PCR and PCR products were analyzed by gel electrophoresis in 1.5% agarose (Promega, Madison, USA) in 0.5XTAEbuffer (40mM Tris, 20mM acetic acid and 1 mM EDTA, pH 8.3) (BIO-RAD). 2-3 drops of ethidium bromide (Biotechnologies, Lot: 3376C425) was used to stain the gel. The gels were visualized under UV transilluminator (Bio-Rad Gel Doc XR+ System, Quantity One software version 4.6.9).

4.3 Task II: Response to Pi limitation and phosphonates bioavailability

4.3.1 Background of strains used in this study

The axenic *N. spumigena* UHCC 0039 (former name, *N. spumigena*AV1) was isolated in 1987 (Sivonen et al. 1989a) from Baltic Sea (Gulf of Finland) and is known to produce toxin (Lehtimäki et al., 2000). The whole genome data from *N. spumigena* UHCC 0039 has become recently available (Teikari et al., 2018b). The genome size of *N. spumigena* UHCC 0039 is 5.39 Mb

The toxic *N. spumigena* UHCC 0060, (axenic, former name, *N. spumigena* HEM) was isolated from the Baltic Sea in the same year as *N. spumigena* UHCC 0039 from Gulf of Finland (Sivonen et al. 1989a) and is genetically similar to *N. spumigena* UHCC 0039.

The non-toxic axenic *N. sphaerocarpa* UHCC 0038 (former name, *N. sphaerocarpa* HKVV) was isolated in 1986 from Baltic Sea (Stockholm archipelago) (Lehtimäki et al., 2000; Fewer et al., 2009). *N. sphaerocarpa* UHCC 0038 was used as negative control strain in growth experiment and alkaline phosphatase activity assay because this strain lacks *phn* gene cluster based on genomic data (unpublished).

4.3.2 Growth experiment and sampling

The growth experiment was performed with above mentioned strains. These strains were originally maintained in continuous batch culture with Z8XS medium under continuous illumination of 3.2-3-7 µmol photons $m^{-2}s^{-1}$. The medium contained 17.1 mg L^{-1} of inorganic phosphate (Pi) and no nitrogen (Kotai, 1972).

Firstly, *Nodularia* cultures were starved in Pi-deplete medium (Z8XS-P) (from here noPi) for 7 days so that the stored intracellular phosphorus was consumed. The cells, in three biological replicates were collected by centrifugation at 7000x g at 20 ºC for 7 min and washed with Z8XS-P medium for three times. Then they were transferred to fresh medium Z8XS-P containing either methylphosphonate (MPn) (Aldrich, Lot: MKBL2916V), ethylphosphonate (EPn) (Aldrich,Lot: MKBP6464V) and 2-aminoethylphosphonate (2APn) (Aldrich, Lot: MKBQ6200V) as a phosphorus source (Figure9).The concentrations of phosphonate compounds were adjusted to be equal to the amount of inorganic phosphate (17.1 mg/L) in the original Z8XS medium. As a positive control, Z8XS medium containing inorganic phosphate (Pi) was used whereas Z8XS-P medium lacking Pi (noPi) was used as negative control. The cultures were grown under continuous illumination of 3.2-3-7 µmol photons $m^{-2}s^{-1}$. Samples, in three

technical replicates were collected every fourth day for determining chl *a* concentration, alkaline phosphatase activity (APA), methane gas concentration and RNA extraction. The cultures were refreshed every 12th day with fresh medium equal to the amount of cultures collected during sampling. The growth experiment for *N. spumigena* UHCC 0060, UHCC 0039 and *N. sphaerocarpa* UHCC 0038 was continuously followed for 53, 50 and 20 days respectively. All media used in growth experiment were prepared in bottles washed with 2 M hydrochloric acid (HCl) to remove adsorbed phosphorus contaminants.

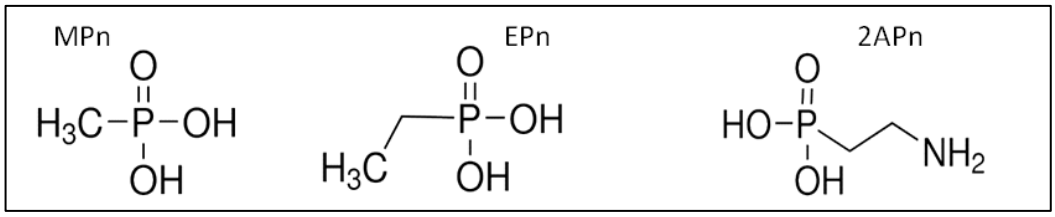

Figure 9 Phosphonate compounds used in this study full form. MPn= methylphosphonate, EPn= ethylphosphonate and 2APn= 2-aminoethylphosphonate.

4.3.3 Chlorophyll *a* – Determination of growth rate

Chl *a* concentration was measured at 4-day intervals during experiment. The collected 1 mL of culture was filtered (Figure 10) using 25 mm glass microfiber filters (GF/C) (GE Healthcare, Cat No. 1822-025) and stored in -80 ºC until extraction. For chl *a* extraction, 1.1 mL of 90% acetone (Thermo Fischer Scientific, United States) was added to frozen samples and samples were mixed by vortex for 30 s and incubated at -20 ºC for 24 h. After 24 h incubation, the samples were mixed again for approximately 1 min and centrifuged at maximum speed for 1 min. Then absorbance was measured at wavelengths 630nm, 647 nm and 665nm by UV-1800 spectrophotometer (Shimadzu Suzhou instruments, Kyoto, Japan). Prior to measurements of samples, 90% acetone was used as blank.

Figure 10 Chlorophyll *a* filtration set-up used in this study.

The concentration of chl *a* was calculated based on the equation provided by Jeffrey and Humphrey (1975). The equation is as follows:

$$\text{Chl } a \text{ (mg/m^3)} = \frac{[11.85\ (D663 - 665) - 1.54\ (D647) - 0.008\ (D630)]\ \text{x v}}{\text{l x V}}$$

where v is volume of acetone (mL), l is cuvette length (cm) and V is volume of filtered water (L).

4.3.4 Alkaline phosphatase activity assay

Extracellular phosphatase activity was measured using the fluorometric method involving hydrolysis of fluorogenic substrate 4-methylumbelliferyl phosphate disodium salt (4-MUD) by measuring the absorbance at 410 nM (Middelboe et al, (1995) (Figure 11). The required standard and substrate for alkaline phosphatase were prepared as following (Appendices, 2A).

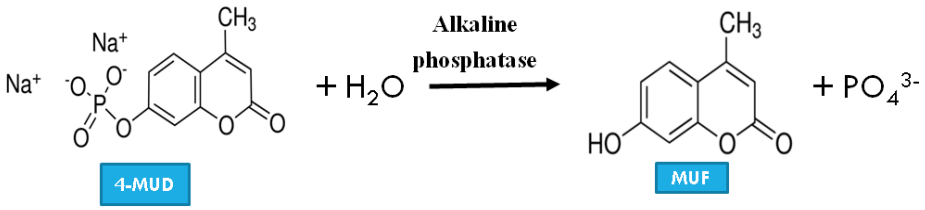

Figure 11 Mechanism of alkaline phosphatase enzyme.

The standard 5mM MUF was diluted in the ratio 1:10 to reach concentration of 0.5mM and 0.05 mM, which was then serially diluted in the ratio 1:2 to obtain concentrations of 5 µM to 78.125 µM. The substrate 5mM 4-MUD was diluted to 25 µM and the samples were diluted in 1:5 ratios. To eliminate background caused by cyanobacteria, the zero samples were prepared by heating samples for 5 min at 100 ºC to inhibit enzyme activity. The assay was carried out in 96-well plate with three technical replicates for each biological replicate. The background sample was prepared by adding 100 µL of substrate and 100 µL of zero samples in one well for each biological replicate. The fluorescence was measured using Victor^3 Plate Reader (Perkin Elmer) at 20 ºC. The enzyme activity (nm/h) was calculated using calibration curve constructed from MUF standards (5 µM – 78.125 µM).

4.3.5 Methane gas measurement

2 mL of cultures of *N. spumigena* UHCC 0060 and UHCC 0039 growing in medium with MPn and Pi was collected and incubated at 20 ºC for 24 h under original growth

conditions in 12 mL Exetainer® vials with Double Wadded Exetainer® Cap (Labco), (Section 4.3.3). Methane emission to the gaseous environment was analyzed using headspace technique (Figure 12).After incubation, the gas samples (8 mL) collected in the headspace were taken using polypropylene syringe attached to a hypodermic needle and injected into helium-washed and vacuumed 3 mL glass vials at atmospheric pressure. As a standard gas, the gas from atmosphere was collected using the same technique. Gas concentration (as parts per million, ppm or cm^3/m^3) was analyzed using an Agilent GC 7890 custom gas chromatography coupled with thermal conductivity, flame ionization and electron capture detectors (Penttilä et al., 2013). The gas concentration obtained was normalized against chl *a* concentration (ppm/mg L^{-1} of chl *a* in culture).

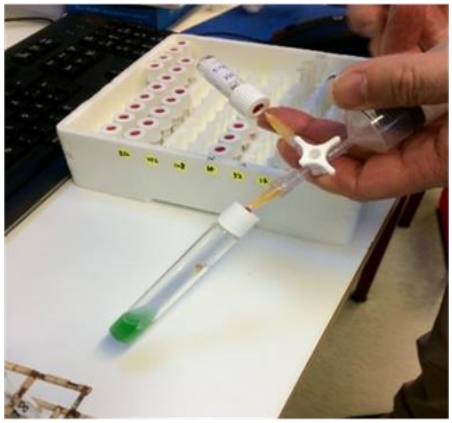

Figure 12 Methane gas sampling by headspace technique (Photo courtesy Jonna Tekari).

4.4 Task III: RT-qPCR assay design and optimization

4.4.1 RNA extraction

The materials and devices used in RNA extraction were cleaned with 70% ethanol followed by RNase AWAY (Molecular BioProducts, Lot: 15530124) to remove endogenous RNAses and exogenous RNAses introduced during sample handling. 5 mL of sample was collected in Rnase free tubes and kept on ice. In order to fix RNA after sampling, 1 mL of fixative, 5% phenol (Lot: 1510003, USA) and 10% ethanol was added immediately. The samples were then filtered through 0.2-micron membrane filters (GE Water and Process Technologies, Catalog number, K02CP04700) and stored at -80 ºC until further use.

Qiagen Rneasy kit was used for RNA extraction. 50 µL of β-Mercaptoethanol (SIGMA, Lot: BCBL6953V) mixed with 5 mL of RLT buffer was used as lysis solution for cells disruption. The autoclaved glass beads of size 425-600 µM (Sigma, Lot: 079K531) and 710-1180 µM (Sigma, Lot: 028K5305V) along with

lysis solution was used for disruption of cells. The steps for RNA extraction were followed as mentioned in the protocol. After eluting RNA, the contaminating DNA was removed by Ambion Turbo Dnase kit. To clean and concentrate RNA, Zymo RNA Clean and Concentrator kit (Catalog no. R1015, Lot: ZRC182683) was used. The concentration of extracted RNA was quantified and the purity and concentrations of RNA samples were checked using Nanodrop ND-1000-spectrophotometer. RNA was considered pure based on the absorbance ratio (260/280 nm ~1.8-2) (Dorak, 2007). The RNA extracts were stored at -80 ºC.

4.4.2 cDNA synthesis: Reverse transcriptase (RT) reaction

Reverse transcriptase (RT) reaction was used for the conversion of total RNA to cDNA. SuperScript® IV First-Strand Synthesis system (Invitrogen) was used for synthesis of cDNA from total RNA. The reaction mixture was prepared in two steps (Table 5).

Table 5 Reverse Transcriptase (RT) reaction setup

Component	**Volume**
10 mM dNTP	1 µL
50 µM random hexamer	1 µL
Template RNA (10 pg- 5 µg total RNA)	upto 11 µL
Nuclease free water	to 13 µL
5x SSIV Buffer	4 µL
100 mM DTT	1 µL
RnaseOUT^{TM} Recombinant RNase Inhibitor	1 µL
Superscript IV Reverse Transcriptase (200 U/L)	1 µL
Total	20 µL

In this experiment, random hexamers were used because the working template is a prokaryotic RNA. At first, RNA-primer mix was prepared with dNTP, random hexamer, template RNA and nuclease free water as mentioned in Table 5and incubated at 65 ºC for 5 minutes followed by on ice incubation for 1 minute. Another reaction tube was prepared mixing 5x SSIV buffer, 100mM DTT, RNase inhibitor and Superscript IV Reverse Transcriptase enzyme. The two reaction tubes were combined and placed in C1000 thermal cycler (BIORAD) that included one cycle of annealing at 23 ºC for 10 minutes, extension at 50-55 ºC for 10 minutes followed by inactivation at 80 ºC for 10 minutes. In order to remove RNA, 1 µL RNase H was added to the combined reaction mixture and incubated at 20 ºC for 20 minutes. The extracted cDNA was not quantified by nanodrop as the concentration of cDNA is generally relative to

the concentration of RNA in the RT-reaction (Applied Biosystems, 2008).

4.4.3 Trial RT-qPCR: Optimization of primer and cDNA concentration

The RT-qPCR assay was designed and optimized for three target genes *pstS, phnD* and *phnJ*. *gyrB* was selected as housekeeping gene for relative quantification of gene expression. RT-qPCR assay was monitored using fluorescent signal detection with CFX96 Touch™ Real time PCR Detection System (BIORAD). PowerUp™ SYBR™ Green Master Mix (ThermoFisher) was used for the procedure.

For RT-qPCR, the primer optimization assay was conducted using two concentrations. 750 nM and 300 nM of both forward and reverse primers were used for trial RT-qPCR to determine the primer combination that produced the lowest Ct value. The high Ct values suggest that there is small number of templates and quantification of small amount would be uncertain. cDNA extracted from *N. spumigena* UHCC 0060 growing in medium containing Pi and MPn was used as template DNA during optimization process. The two concentrations of cDNA, 2 ng and 10 ng were additionally used during trial qPCR to find optimum cDNA concentration. Each primer combination was set up in triplicates. Reaction master mix setup and cycling parameters for trial RT-qPCR are shown (Table 6 and 7 respectively).

Table 6 Reaction mixture for primer and cDNA concentration optimization

RT-qPCR mix	**Volume**
SYBR green master Mix	10 µL
Forward Primer (750 nM/ 300 nM)	0.3 µL
Reverse Primer (750 nM/ 300 nM)	0.3 µL
cDNA template (2 ng/ 10 ng)	1 µL
Nuclease free water	8.4 µL
Total	20 µL

Table 7 RT-qPCR cycling parameters during trial run

Phase	**Conditions**
1. Initial denaturation	95 ºC for 7 min
2. Denaturation	95 ºC for 10 s
3. Annealing/Extension + plate read	60.5 ºC for 30 s
4. Phase 2-3 Repeats	39 repeats
5. Final Elongation	95 ºC for 10 s
6. Melt curve + Plate read	65 ºC to 95ºC
7. Hold	4 C

4.4.4 Experimental RT-qPCR

The experimental RT-qPCR was set up with optimized primers and cDNA. Based on results obtained from growth experiment and alkaline phosphatase activity assay, the samples for transcriptomic studies were collected at day 12 and 24 from cultures growing in noPi, Pi, MPn and 2APn. The RT-qPCR reaction mix consisted of 10 ng template cDNA, 300 nM of both primers, and 10 µl of Power Up^{TM}SYBR Green Master Mix (Thermo Fisher Scientific) in a total volume of 20 µL. Purified water was used as a no template control. The qPCR cycling parameters and annealing temperature for melting curve was similar to trial RT-qPCR (Table 7). Three technical replicates for each biological replicate were used.

4.4.5 Relative quantification of experimental RT-qPCR data

The relative gene expression was determined by Comparative Ct method ($2^{-\Delta\Delta Ct}$), also known as Livak method (Livak and Schmittgen, 2001). The required Ct values to determine the relative expression of one target gene in a test sample and calibrator sample using a reference gene as normalizer is shown (Table 8).The steps of calculations are explained below (Table 9). In this study, the Ct values of *gyrB* obtained in Pi were used as calibrator for the calculation of relative expression of target genes *phnJ*, *phnD* and *pstS* in noPi (as negative control) and MPn and 2APn (treatments).

Table 8 Required Ct values for relative quantification using reference genes as normalizers

Gene	**Test**	**Calibrator (cal)**
Target (tar)	$Ct_{(tar, test)}$	$Ct_{(target,cal)}$
Reference (ref)	$Ct_{(ref,test)}$	$Ct_{(ref,cal)}$

Adapted from BIO-RAD (2006)

Table 9 Steps for calculation of relative gene expression by $2^{-\Delta\Delta Ct}$method

Step I The Ct values of the target gene is normalized to that of reference gene for both test and calibrator sample $\Delta Ct_{(test)} = Ct_{(tar, test)} - Ct_{(ref, test)}$ $\Delta Ct_{(cal)} = Ct_{(tar, cal)} - Ct_{(ref, cal)}$
Step II The ΔCt of the test sample is normalized to the ΔCt of the calibrator to obtain ΔΔCt. $\Delta\Delta Ct = \Delta Ct_{(test)} - \Delta Ct_{(cal)}$
Step III The fold change in gene expression is calculated as: Fold change = $2^{-\Delta\Delta Ct}$

4.4.6 Efficiency of experimental RT-qPCR

The standard curves were generated for each primer pairs during experimental RT-qPCR. The standard curves were constructed from the 10-fold serial dilutions of genomic DNA (gDNA) of *N. spumigena* UHCC 0060 (5.38 Mbp; unpublished) growing in inorganic phosphorus (Pi) containing medium. The different dilutions were made to contain 10^6 to 10^1 copies of gDNA in a reaction. The amplification efficiency (E) for each primer pair was calculated from regression slope of the standard curve using equation:

$$\text{Efficiency(E)} = \left(10^{-\frac{1}{\text{slope}}} - 1\right) \text{x } 100\%$$

An efficiency of 1 means a doubling of product in each cycle and the slope of the standard curve is -3.32 in this case. Efficiency between 90%-105% is generally considered good in RT-qPCR (Bio-Rad, 2006).

5. RESULTS

5.1 Detection of *phn* gene cluster

5.1.1 Identification of *phn* gene cluster in *Nodularia* strains

Bioinformatic analysis of *phn* gene cluster revealed that all three sequenced strains of *N. spumigena* carried complete *phn* gene cluster (Figure 13, lower panel). The *phn* gene cluster in *N. spumigena* UHCC 0039, CCY9414 were found to span a region of ~12.5 kb strains and ~10.6 kb for *N. spumigena* CENA 596 strain. The *phn* gene cluster of *N. spumigena* UHCC 0039 (Figure 13, upper panel) consisted of 14 genes similar to *phn* operon in *E. coli* (Figure 3). The cluster was located at 535027-547520 bp and consisted of phosphonate transporter *phn*(*C-E12*), regulatory gene *phnF* and C-P lyase complex *phn*(*G-M*). The remaining two genes may be accessory genes. The gene cluster between the Baltic Sea *N. spumigena* UHCC 0039 and its counterpart *N. spumigena* CCY9414 showed clear synteny. The organization of *phn* gene cluster in *N. spumigena* CENA596 was different from the Baltic Sea *N. spumigena* strains (Figure 13, B). Overall, the *phn* operon consists of phosphonate transporter followed by C-P lyase genes.

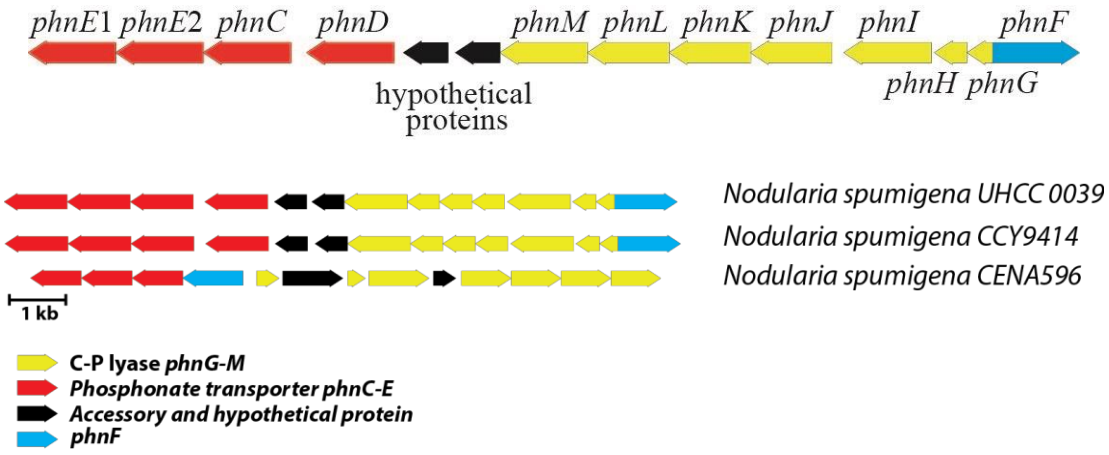

Figure 13 Schematic representation of *phn* gene cluster in *N. spumigena* UHCC 0039 (upper panel) and comparison of *phn* gene cluster between available genomes of *N. spumigena* strains (lower panel).

5.1.2 Primer design and PCR optimization

Primer sets used in this study for genes *phnD* (Figure 14, A), *phnJ* (Figure 14, B), *pstS* (Figure 14, C) and *gyrB* (Figure 14, D) are completely complementary to the priming sites in *N. spumigena* strains UHCC 0039, UHCC 0060, CCY9414 and CENA 596. The

priming sites of *phnD* (Figure 14, A) had however mismatches with gene sequences in these *Nodularia* strains.

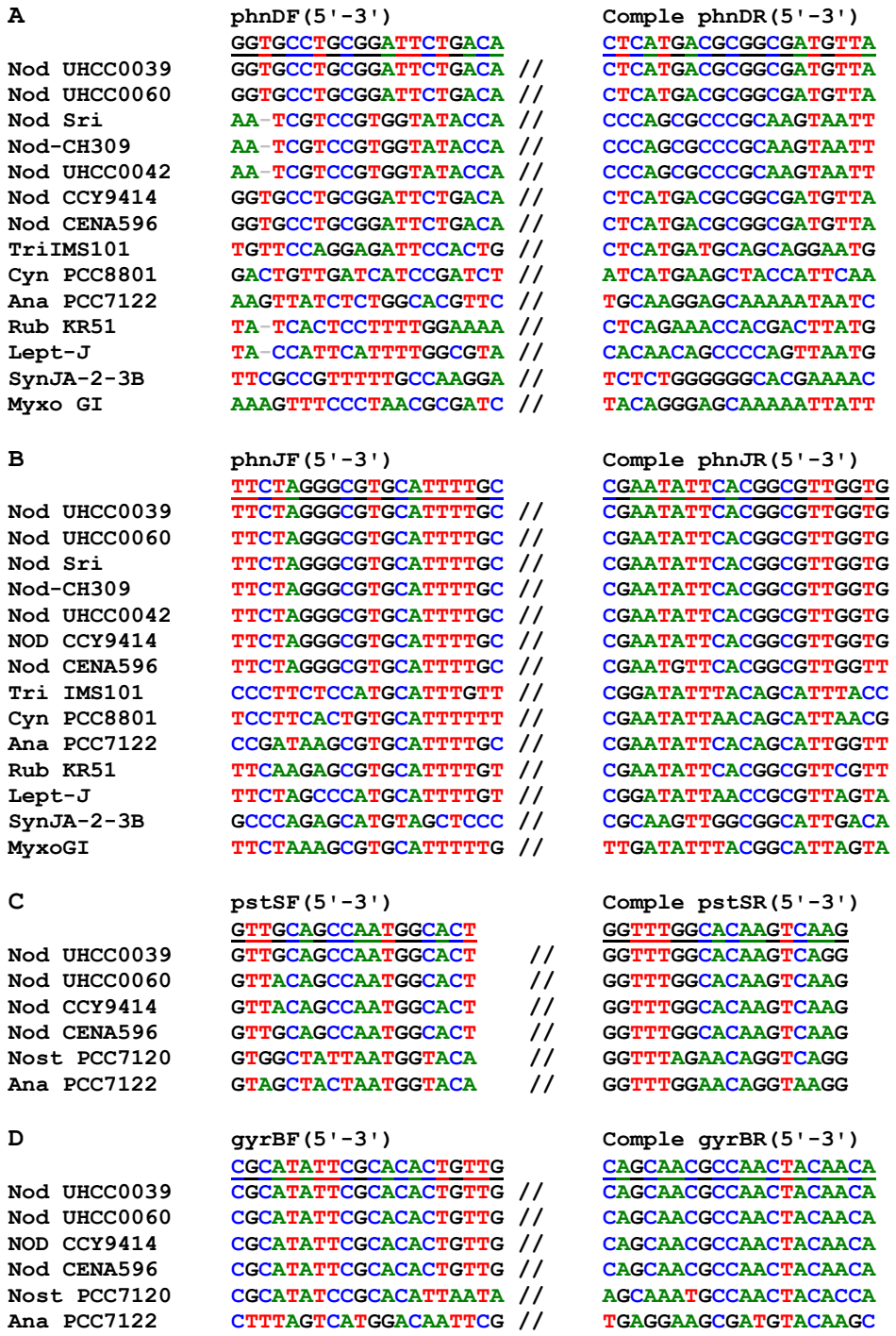

Figure 14 The annealing sites for primer pairs designed in this study for *phnD* and *phnJ* of *phn* gene cluster, *pstS* and *gyrB* genes of representative cyanobacterial strains. Complementary sequences are shown for reverse primers (true primer sequences can be found in Table 2). The region between priming sites are denoted by //. A: alignment sites for *phnD*F and *phnD*R, B: alignment sites for *phnJ*F and *phnJ*R, C: alignment sites for *pstS*F and *pstS*R and D: alignment sites for *gyrB*F and *gyrB*R.

The designed *phnJ* primer pair (Figure 14, B) was also complementary to other *Nodularia* strains (*Nodularia* sp. Sri, *Nodularia* sp. CH309 and *Nodularia* sp. UHCC 0042) used from in-house blast server. In addition, there were differences between all the primers and annealing sites in cyanobacterial strains used from NCBI ensuring that primers designed here are *N. spumigena* specific.

Gradient PCR (Figure 15) was performed to find the optimal annealing temperature from the range of 50-60 ºC for *N. spumigena* UHCC 0060 with primers designed for genes *phnJ* (Figure 15, A), *phnD* (Figure 15, B) and *gyrB* (Figure 15, C). All annealing temperatures amplified the correct PCR product for all primer sets. *phnJ* gene was however, maximally amplified at higher annealing temperatures from 56 to 60°C. For conventional PCR, 60.5 ºC was selected as annealing temperature.

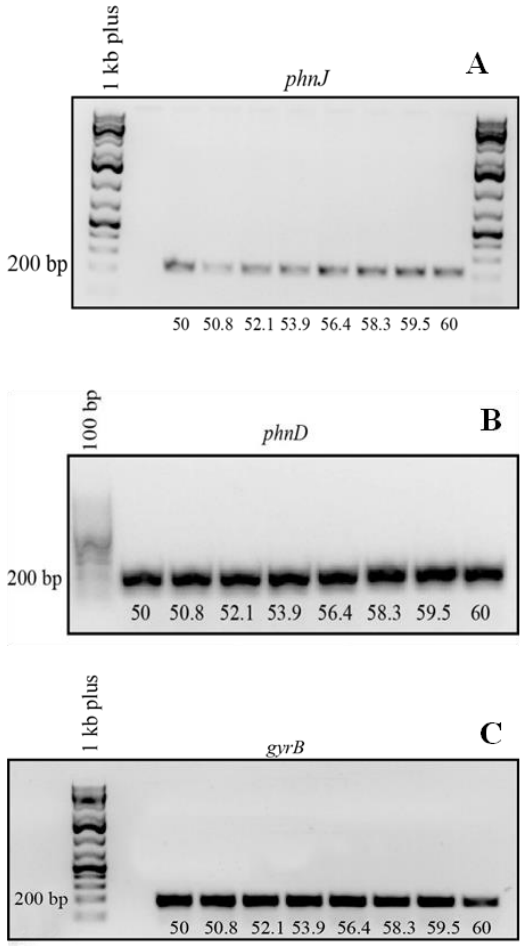

Figure 15 Gel images of gradient PCR showing amplification of genes: *phnJ* (A), *phnD* (B), and *gyrB* (C) with expected product sizes 216 bp, 225 bp, and 189 bp respectively. Gradient PCR with annealing temperature (50-60 ºC) on 1.5% ethidium bromide stained agarose gel. Running conditions: 120 V, 30 min. 1 kb plus and 100 bp DNA ladder (ThermoScientific).

The specificity of designed primers was checked by conventional PCR in DNA samples from three *Nodularia* strains and a *Dolichospermum* sp. strain to amplify

phnJ, *phnD*, *pstS* and *gyrB* genes (Figure 16). The primer pair for *phnJ* amplified an ~216 bp fragment only in *N. spumigena* UHCC 0060, *N. spumigena* UHCC 0039 and *N. spumigena* UHCC 0040 (Figure 16, A). The *phnJ* gene was not amplified from *N. sphaerocarpa* UHCC 0038.

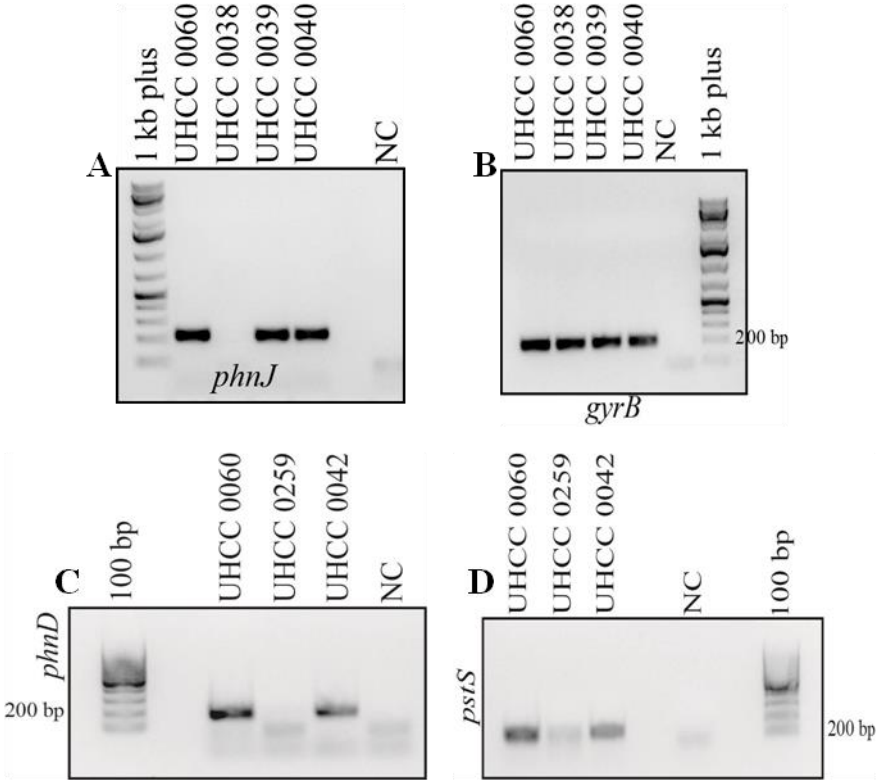

Figure 16 Conventional PCR screening of the genes *phnJ* (A) and *gyrB* (B) in *N. spumigena* UHCC 0060, *N. sphaerocarpa* UHCC 0038, *N. spumigena* UHCC 0039 and *N. spumigena* UHCC 0040 (upper panel).Amplifications of the genes *phnD* (C) and *pstS* (D) in *N. spumigena* UHCC 0060, *Dolichospermum* sp. UHCC 0259 and *N. spumigena* UHCC 0042 (lower panel). NC stands for negative control. 1.5% agarose gel stained with ethidium bromide. Running conditions: 120 V, 30 min. 1 kp plus and 100 bp DNA ladder (Thermo Scientific). Refer to Table 3 for more information of *Nodularia* sp. strains.

The housekeeping gene *gyrB* (~189 bp) was amplified in all tested *Nodularia* strains (Figure 16, B). The amplification of ~ 225 bp *phnD* gene (Figure 16, C) and ~119 bp *pstS* gene (Figure 16, D) were observed in *N. spumigena* UHCC 0060 and *N. spumigena* UHCC 0042. The amplification of *phnD* and *pstS* genes was relatively weaker in *Nodularia* sp UHCC 0042. In *Dolichospermum* sp. UHCC 00259, the *phnD* gene amplification was not found but very low amplification of *pstS* gene was observed (Figure 16, D). Therefore, the primer pairs designed here were considered to be specific to *Nodularia* genus and were selected for further analysis in screening *phn* gene cluster from UHCC cyanobacterial strains isolated from the Baltic Sea.

5.1.3 Molecular detection of *phn* gene cluster in UHCC strains

The amplified PCR products with correct sizes for *phnD* (upper panel) and *phnJ* (lower panel) genes are shown in Figure 17. The *N. spumigena* specific primers designed for *phnD* and *phnJ* genes amplified both genes from all tested *N. spumigena* strains. *N. sphaerocarpa* UHCC 0038 was a negative control strain and as expected, *phnJ* and *phnD* primers did not amplify any DNA region of this strain. *phnD* and *phnJ* genes were not amplified from other cyanobacterial strains including *N. sphaerocarpa* UHCC 0052, *Aphanizomenon flos-aquae* UHCC 0183 and *Nodularia* sp. UHCC 0075. However, amplification was observed for *phnD* and *phnJ* genes in *Nodularia* sp. UHCC 0099 and UHCC 0158.

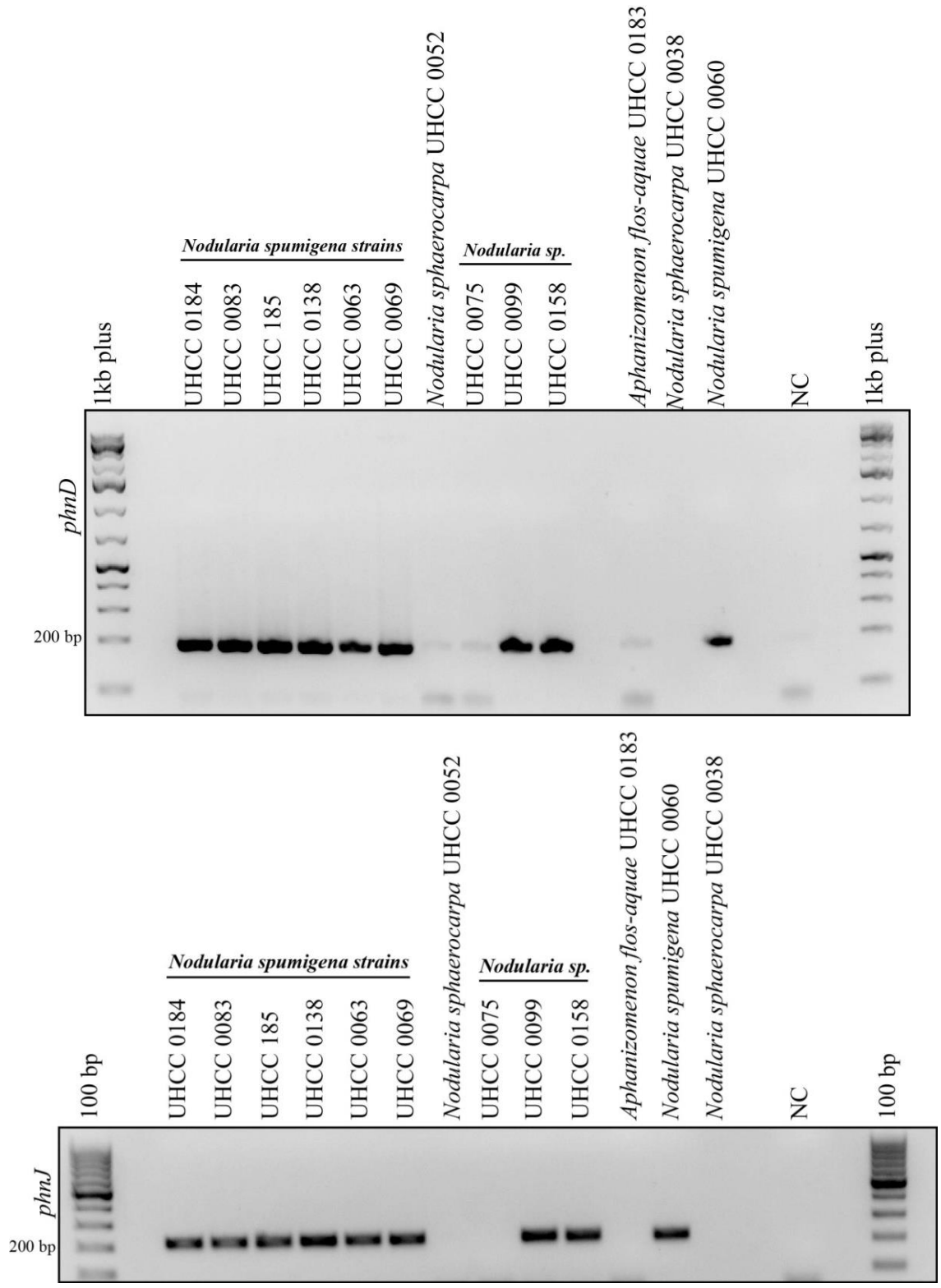

Figure 17 Screening for *phnD* (225 bp) (upper panel) and *phnJ* (216 bp) (lower panel) genes from different cyanobacteria isolated from the Baltic Sea. *N. spumigena* UHCC 0060 as positive control and *N. sphaerocarpa* UHCC 0038 as negative control. NC, negative control; 100 bp plus ladder (ThermoScientific).

5.2 Phosphonates as sole phosphorus source during Pi limitation

5.2.1 Growth in different phosphonate compounds

Growth pattern of all three *Nodularia* strains were assessed in phosphonate treatments (MPn, EPn, 2APn) and Pi and noPi after seven-day starvation (Figure 18). The growth increased in both *N. spumigena* UHCC 0039 and UHCC 0060 prior to addition of fresh media for each treatment on 12th day. The growth slowed down in both strains until day 16as shown by chl *a* concentration in all treatments. After day 16, *N. spumigena* UHCC 0039 and UHCC 0060 started displaying growth based on preferable form of phosphorus. The growth was elevated in both strains in medium with Pi and decreased in noPi as expected. Nevertheless, very minor growth was observed in noPi suggesting that the intracellular polyphosphate storage granules were not completely depleted. The interesting finding was that both tested strains were able to grow in medium containing MPn after preferable Pi.

In *N. spumigena* UHCC 0039 (Figure 18, A), the highest chl *a* concentration (6.60 mg/L of culture) was found on day 46 in medium containing MPn. Whereas, the highest chl *a* concentrations observed in Pi containing medium was 9.76mg/L on day 53. In addition, minor growth was observed in medium with EPn. However, *N. spumigena* UHCC 0039 was not able to use 2APn for long.

In *N. spumigena* UHCC 0060 growing in MPn, the maximum chl *a* concentration (4.52 mg/L) was found on day 43. While, the highest chl *a* concentration observed in Pi medium was 5.92 mg/L on day 43. Additionally, 2APn appeared to be a suitable phosphorus source after Pi and MPn. However, EPn was not a preferable phosphorus source for *N. spumigena* UHCC 0060 (Figure 18, B) as shown by the visible cell chlorosis.

In case of *N.sphaerocarpa* UHCC 0038 (Figure 18, C), maximum growth was observed in Pi medium. *N. sphaerocarpa* UHCC 0038 could not grow as well on phosphonate containing medium as on Pi containing medium indicated by the bleached cells on phosphonate treatments. Further, the chl *a* concentrations were remarkably different between *N. sphaerocarpa* and strains of *N. spumigena*. The maximum chl *a* concentration in *N. sphaerocarpa* was found to be 1.20 mg/L of culture growing in Pi on day 20.

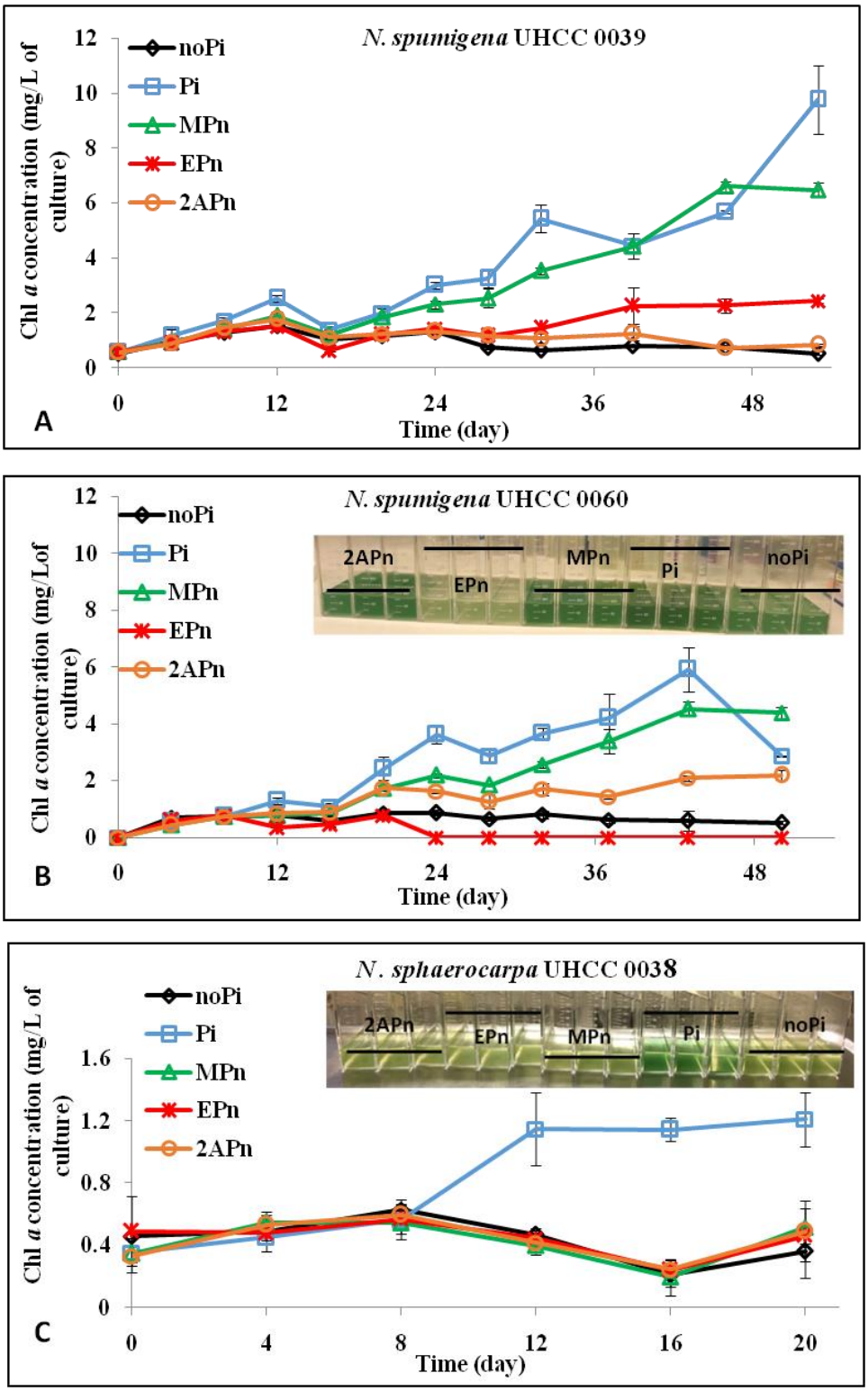

Figure 18 Growth of *N. spumigena* UHCC 0039 (A), UHCC 0060 (B) and *N. sphaerocarpa* UHCC 0038 (C) expressed in Chl *a* concentration (mg/L of culture). noPi= without phosphorus, negative control; Pi= inorganic phosphate, positive control; MPn= Methylphosphonate; EPn= ethylphosphonate and 2APn= 2-aminoethylphosphonate. Error bars indicate mean ± SE

5.2.2 Alkaline phosphatase activity (APA)

The maximum alkaline phosphatase activity was found in noPi medium in studied strains *N. spumigena* UHCC 0039 (Figure 19, A) and UHCC 0060 (Figure 19, B) and in Pi medium in *N. sphaerocarpa* (Figure 19, C). No elevated APA was observed in Pi medium in *N. spumigena* strains.

The maximum enzyme activity observed in noPi in *N. spumigena* UHCC 0039 was found to be approximately 45000 nm/h on day 20 when chl *a* concentration was 1.16 mg/L of culture. In addition, there was notable increase in APA in medium containing 2APn in *N. spumigena* UHCC 0039. The highest APA in 2APn was found on day 20 (41000 nm/h).Surprisingly, no increasing APA was observed in medium containing MPn, although, the medium lacked Pi.

N. spumigena UHCC 0060 showed similar trend as *N. spumigena* UHCC 0039 in APA. The highest APA (37000 nm/h) was found in noPi medium on day 20 at 0.84 mg/L chl *a* concentration. Increased APA was also observed in 2APn but it was lower compared to UHCC 0039. And, APA was not found to be elevated in medium with MPn.

N. sphaerocarpa UHCC 0038 (Figure 19 C) showed less activity than the other two tested strains (note different scales). The APA was highest in Pi medium followed by noPi medium. The maximum enzyme activity was found to be approximately 2700 nm/h in Pi medium in day 20.

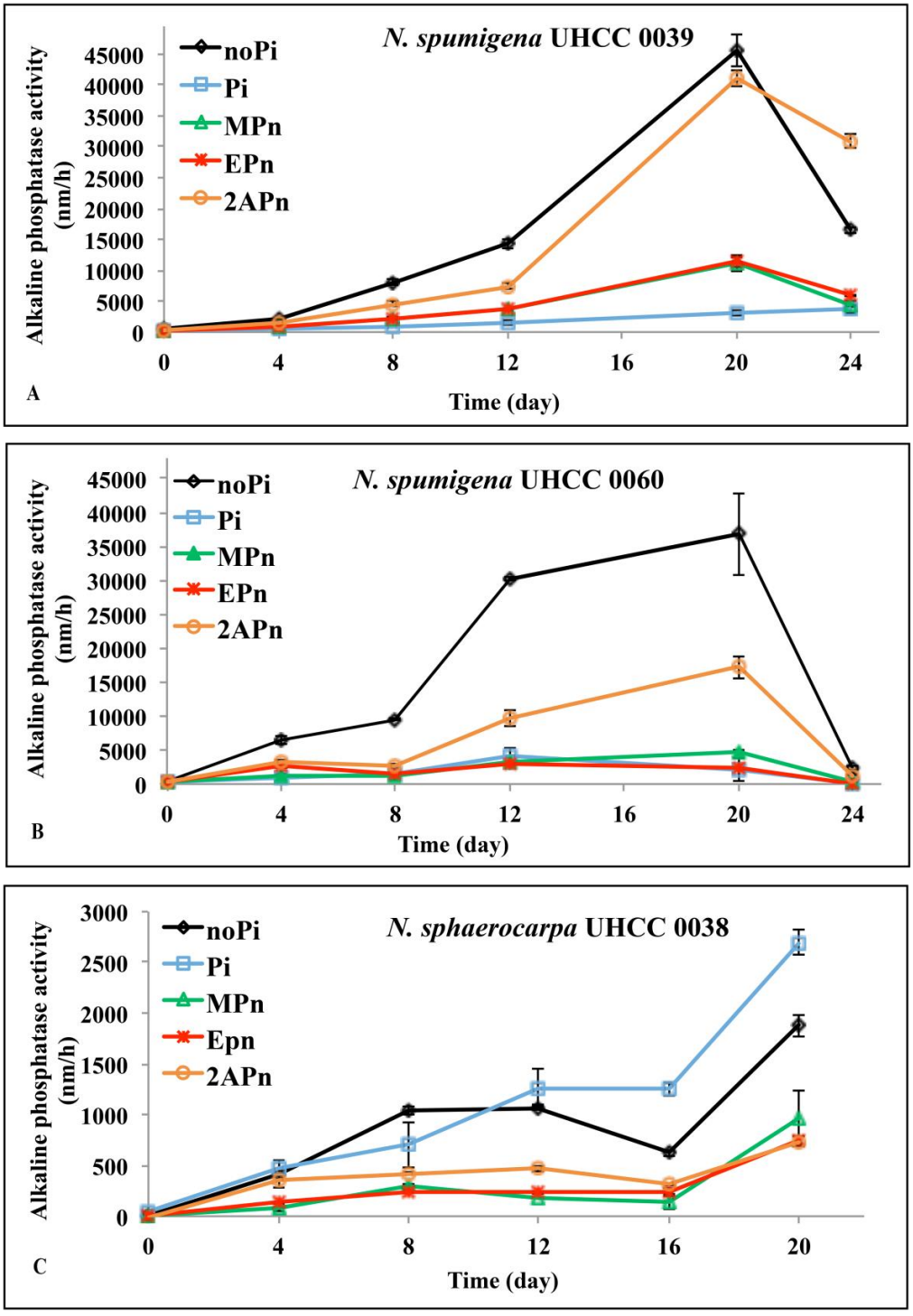

Figure 19 Alkaline phosphatase activity in *N. spumigena* UHCC 0039 (A), UHCC 0060 (B) and *N.sphaerocarpa* UHCC 0038 (C). noPi= without phosphorus, negative control; Pi= inorganic phosphate, positive control; MPn= Methylphosphonate; EPn= Ethylphosphonate and 2APn= 2-aminoethylphosphonate. Error bars indicate mean ± SE.

5.2.3 Methane gas liberation by *N. spumigena* UHCC 0039 and UHCC 0060

Methane gas liberated from MPn was found to be of varying concentrations in different growth phases of both strains *N. spumigena* UHCC 0039 (Figure 20, A) and *N. spumigena* UHCC 0060 (Figure 20, B). It was clearly visible that the methane gas was not released from Pi containing medium in *N. spumigena* UHCC 0039 and UHCC 0060 compared to MPn. Overall, *N. spumigena* UHCC 0060 appeared to be liberating more methane compared to *N. spumigena* UHCC 0039 from MPn.

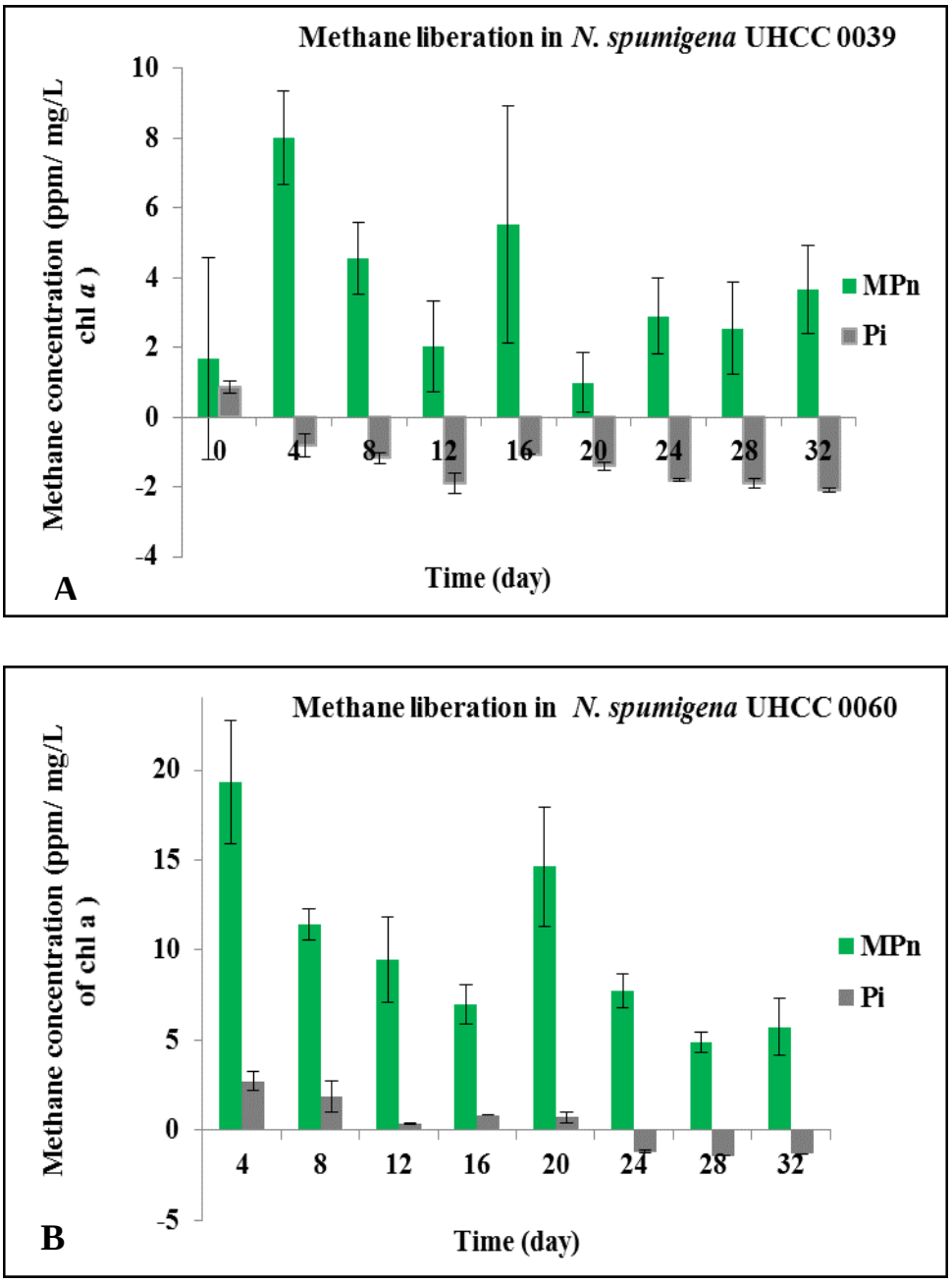

Figure 20 Methane gas liberation by *N. spumigena* UHCC 0039(A) and UHCC 0060 (B) in medium containing MPn and Pi. The gas concentration is normalized against chl *a* concentration in mg/L of culture. Error bars indicate mean ± SE

5.3 RT-qPCR design and optimization

5.3.1 Primers and cDNA testing during trial RT-qPCR

The different concentrations of primers and cDNA were additionally tested (Table 10) by RT-qPCR. The average Ct values for 2ng of cDNA were higher compared to 10 ng of cDNA in condition with inorganic phosphate (Pi). The primer combination with 750 nM of both forward and reverse primer amplified all tested genes with higher Ct values compared to 300 nM of primer pair.10 ng of cDNA with 300 nM of both forward and reverse primer produced lower Ct values. There was no amplification with this combination in NTC. Hence, 10 ng of cDNA with 300 nM primer were used for experimental RT-qPCR assay.

Table 10 Amplification as average Ct values of three reactions obtained during trial RT-qPCR for each gene

	phnD		***phnJ***		***pstS***	
cDNA/primer(Fwd /Rev)concentration	Pi	NTC	Pi	NTC	Pi	NTC
2 ng/750 nM	29.86	35.32	30.19	36.87	31.16	30.43
10 ng/750 nM	27.22		16.02		27.3	
10 ng/300 nM	Nt	NA	29.38	NA	26.01	NA

Pi: Inorganic phosphate; NTC: No template control; NA: No amplification and Nt: Not tested

5.3.2 Experimental RT-qPCR specificity and performance

5.3.2.1 Efficiency by standard curve

The standard curves generated for each primer pairs amplifying genes *phnD*, *phnJ*, *pstS* and *gyrB* in experimental RT-qPCR is shown Figure 21. The efficiency of amplifications for all primer pairs was determined from regression slope of standard curves (Figure 21). Correlation coefficients (R^2) were calculated to be 0.99 for all primer pairs. The efficiency of all primers ranged from 98.8%-100.5 % which lie within the accepted range stated by BIORAD (Table 11, Appendices 3A) (BIORAD, 2006).

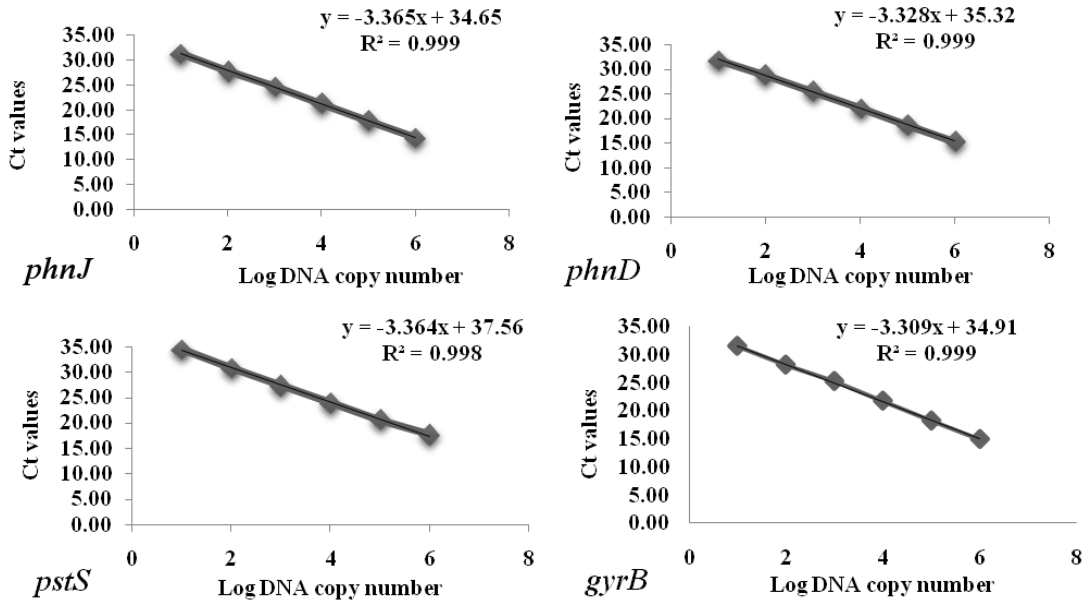

Figure 21 Standard curves showing efficiency of primer pairs designed for genes *phnJ*, *phnD*, *pstS* and *gyrB*. The graphs were constructed by plotting the Ct values relative to log of DNA copy number.

5.3.2.2 Melting curve analysis

In addition, analysis of melting curve for all primers showed a single peak indicating amplification of desired amplicon only. Melting temperature for phnJ and phnD primers was found to be 82.5 ºC and 80.5ºC respectively in *N. spumigena* UHCC 0039 (Figure 22). The melting temperature varied between 78.5-79ºC for pstS and gyrB primers.

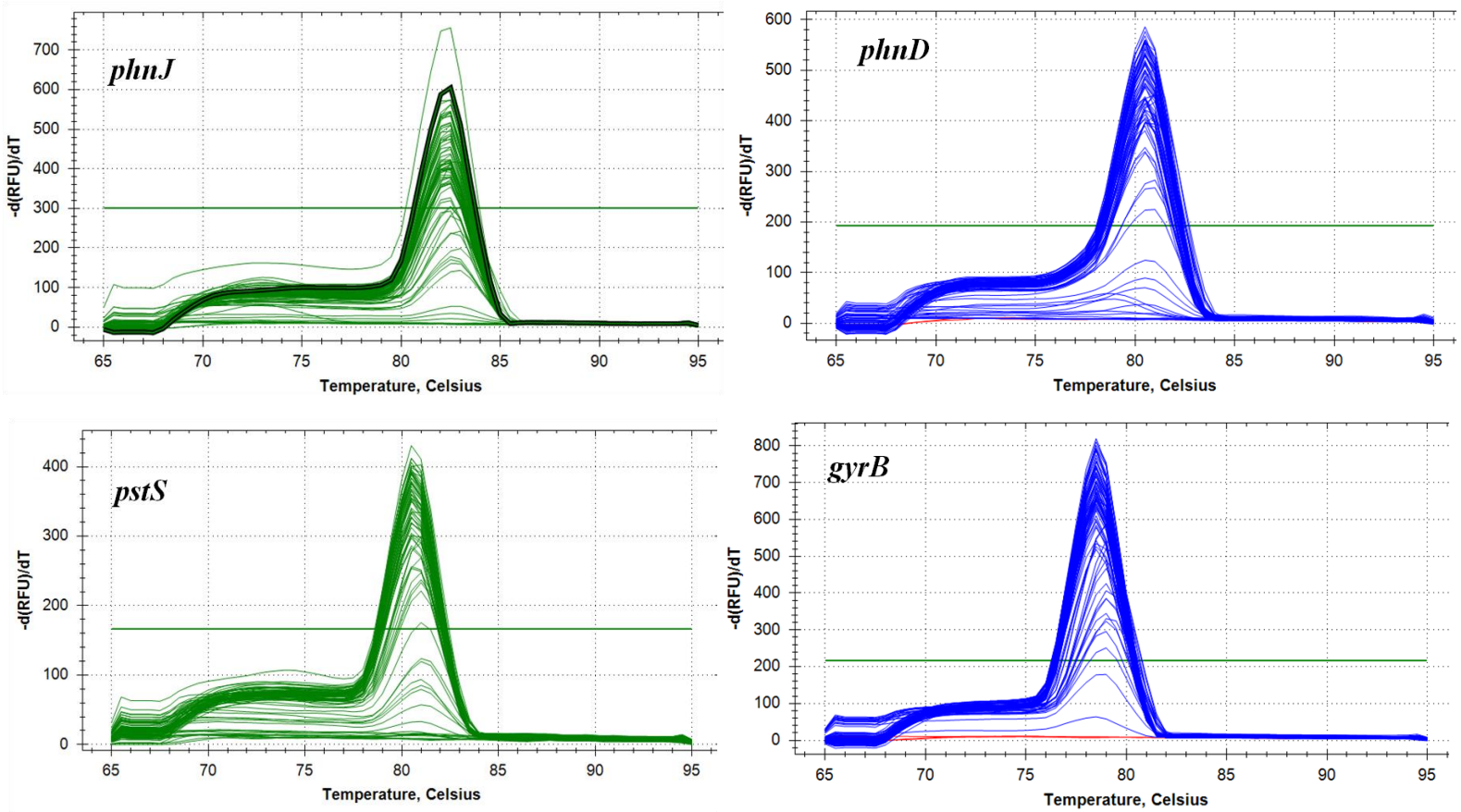

Figure 22 Melting curve obtained from amplification of primers used for *phnJ*, *phnD*, *pstS* and *gyrB* genes in *N. spumigena* UHCC 0039 by RT-qPCR. Red peak indicates melting temperature of NTC (no template control).

In *N. spumigena* UHCC 0060 (Figure 23), the melting temperature with phnJ primers was found to be precisely 82.5 ºC. The melting temperature for phnD primers was 80.5 ºC and ranged between 80.5-81 ºC for pstS primers. No melting peak was observed in NTC as shown by red peak in Figure 22 and 23 in both strains.

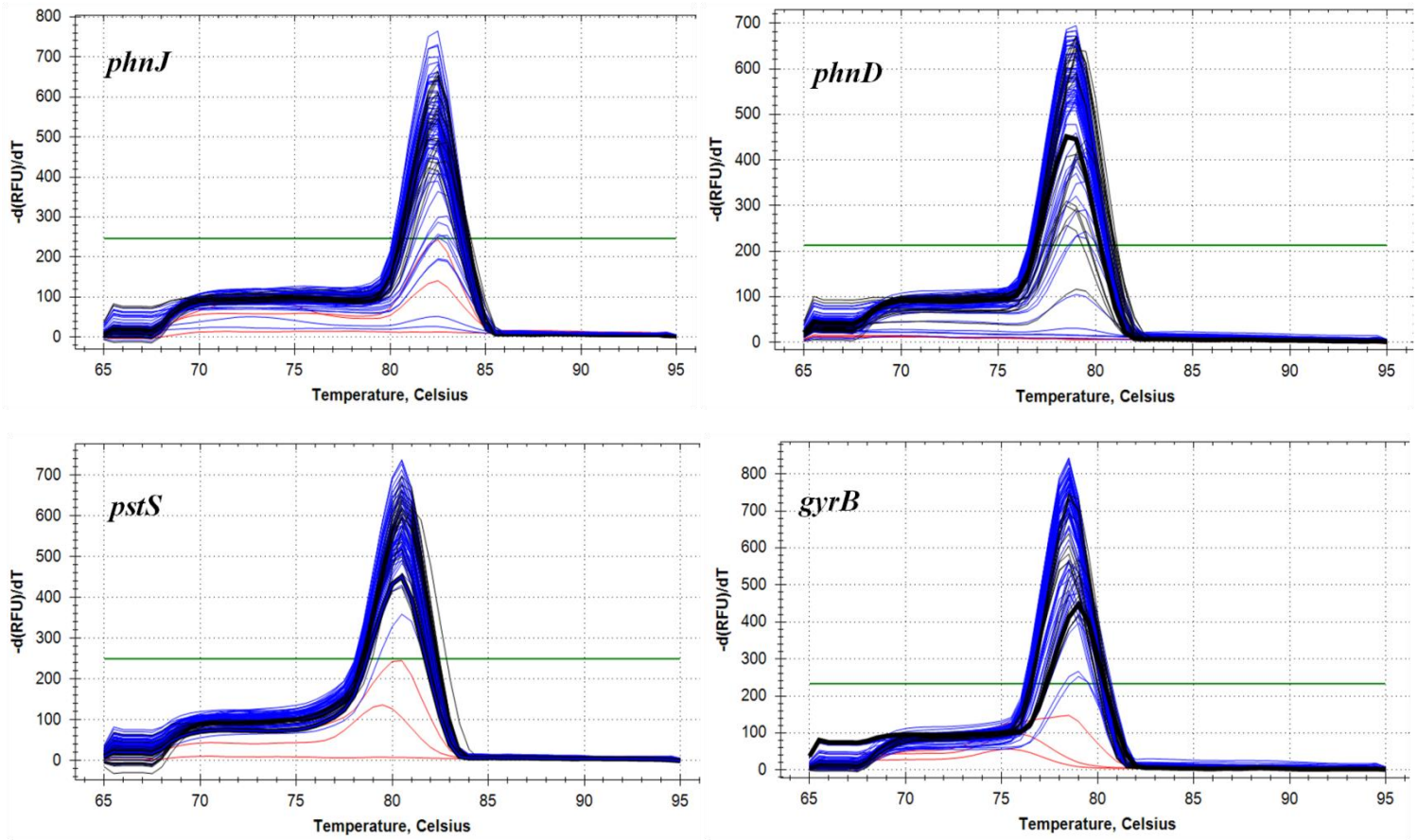

Figure 23 Melting curve obtained from amplification of primers used for *phnJ, phnD, pstS* and *gyrB* genes in *N. spumigena* UHCC 0060 by RT-qPCR Red peak indicates melting temperature of NTC (no template control)

5.3.2.3 Stability of *gyrB* gene

Figure 24 shows the average Ct values of *gyrB* gene on day 12 and 24 in different growth media. The expression of *gyrB* gene was almost constant in both controls (no Pi and Pi) and treatments (MPn and 2APn).

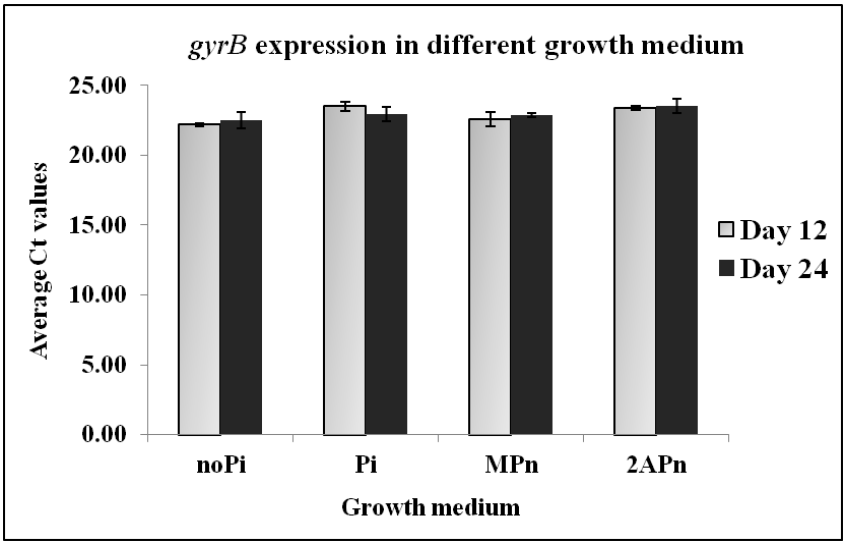

Figure 24 Abundance of *gyrB* transcripts in *N. spumigena* UHCC 0060 in Pi (inorganic phosphate), noPi (without phosphorus), MPn (Methylphosphonate) and 2APn (2-aminoethylphosphonate). Error bars calculated from mean of three Ct values± SE.

5.4 Transcripts abundance of *phnD, phnJ* and *pstS* based on experimental RT-qPCR

The transcriptional levels of *phnJ*, *phnD* and *pstS* genes of *N. spumigena* strains UHCC 0039 and UHCC 0060 in different growth conditions are shown in Figure 25. The relative expression of the target genes were calculated as fold-change compared to housekeeping gene *gyrB* in Pi.

In *N. spumigena* UHCC 0039 (Figure 25, A), presence of MPn showed a high upregulation of *phnJ* (~190-fold) on day 12, which remained stable until day 24 (~110-fold). In 2APn containing medium, the expression of *phnJ* was very high on day 12 (~455-fold) which drastically decreased to ~20-fold on day 24. *phnJ* gene expression was almost undetectable in noPi.

The *phnD* gene was highly upregulated (~2000-fold) on day 12 in 2APn which greatly decreased to ~485-fold on day 24. In noPi medium, *phnD* was ~600-fold upregulated on day 12 and increased to ~940-fold on day 24. There was very minor

upregulation of *phnD* gene in MPn on day 12 compared to noPi and 2APn (Figure 25, C).

pstS showed expression fold level to be low compared to other two studied genes *phnD* and *phnJ* (note different scales) (Figure 25, E and F). In noPi medium, *pstS* gene was found only to be 0.98-fold upregulated in day 12 and it increased to 1.04-fold on day 24. In medium containing MPn and 2APn, *pstS* gene expression was less than 1-fold upregulated on both day 12 and 24 (Figure 25, E).

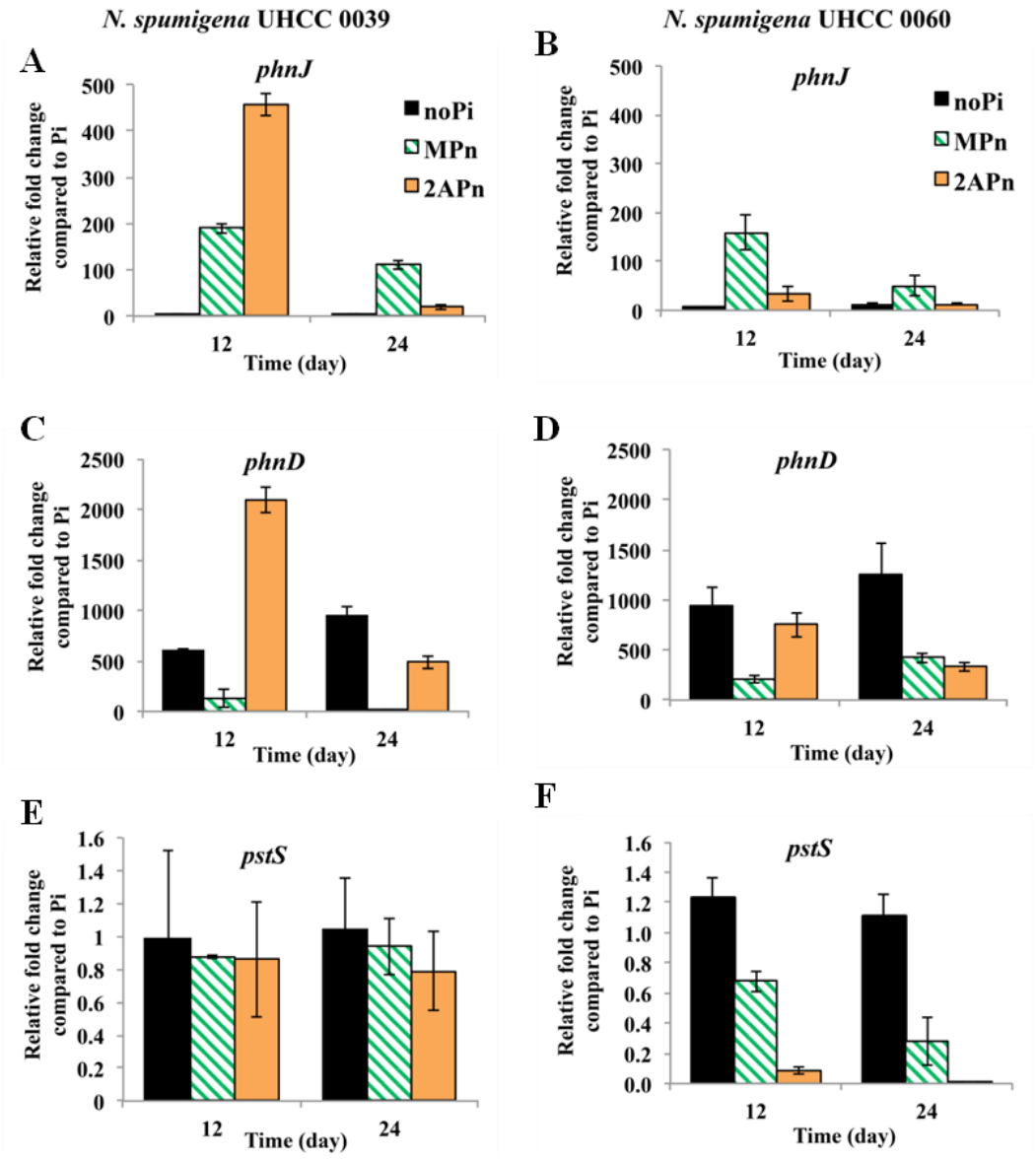

Figure 25 Relative abundance of transcripts *phnJ*, *phnD* and *pstS* obtained from RT-qPCR in *N. spumigena* UHCC 0039 (right panel) and UHCC 0060 (left panel) compared with Pi. noPi= without phosphorus; Pi(inorganic phosphate); MPn (Methylphosphonate); and 2APn (2-aminoethylphosphonate). Error bars calculated from mean of three Ct values± SE

Similarly, in case of *N. spumigena* UHCC 0060, *phnJ* gene had similar gene expression pattern (Figure 25, B). *phnJ* exhibited higher expression (~160-fold) on day 12 and remained more or less stable until day 24 (50-fold) in presence of MPn medium. *phnJ* gene was relatively less expressed in 2APn on both days. The expression of *phnJ* was ~33-fold high on day 12 that decreased to ~12-fold on day 24. In case of noPi, *phnJ* gene showed very low expression.

The *phnD* gene was highly upregulated (~753-fold) on day 12 in 2APn, which decreased to ~331-fold on day 24. In noPi medium, *phnD* gene was ~945-fold upregulated on day 12 and expression elevated to ~1250-fold on day 24 (Figure 25, D). There was minor upregulation of *phnD* gene in MPn compared to noPi and 2APn in both strains.

In noPi medium, *pstS* gene was found to be only ~1.23-fold upregulated on day 12 that decreased to ~1.11-fold on day 24. The *pstS* gene expression was less than 1-fold upregulated on day 12 and 24 in MPn and 2APn in *N. spumigena* UHCC 0060 (Figure 25, F).

6. DISCUSSION

One of the environmental stresses includes the limitation of essential macronutrient Pi. Usually, marker proteins are required to detect the nutrient limitation at the level of the individual cell in a given environment (Dignum et al., 2005). For Pi limitation, two-component signaling system known as *pho* regulon has been studied in cyanobacteria (Hirani et al., 2001; Suzuki et al., 2004). However, Su et al., (2007) have shown the presence of genes related to carbon fixation, nitrogen assimilation and photosynthesis in cyanobacteria in the *pho* regulon besides genes involved in phosphorus metabolism and these genes were regulated Pi availability. Therefore, it has been complicated to find a suitable marker for Pi limitation in cyanobacteria.

Molecular based detection methods are valuable to monitor phosphorus and phosphonate status in the Baltic Sea because the bioavailability of phosphorus influences cyanobacterial blooms. Moreover, harmful toxic diazotrophic *N. spumigena* has been found to be successfully flourishing despite being in Pi limited environment and mechanisms behind that ability still need to be investigated. A PCR based assay was developed for the first time in this study to detect the presence and expression of genes induced during Pi limitation and phosphonates bioavailability in the laboratory grown cyanobacterial strains of the Baltic Sea. The results obtained from this study thus provide new genetic markers to test the availability of phosphonates and Pi limitation in similar environments.

6.1 Distribution of *phn* operon in cyanobacteria

The sporadic distribution of *phn* gene cluster in cyanobacteria has been shown before because of horizontal gene transfer, which is a common phenomenon in genetically related cyanobacteria (Dyrhman et al., 2006; Villarreal-Chiu et al., 2012; Choi et al., 2013). In this study, bioinformatics analysis of previously sequenced *N. spumigena* CCY9414 and *N. spumigena* CENA 596 (Popin et al., 2016) and *N. spumigena* UHCC 0039 (Teikari et al., 2018a) showed the presence of full *phn* gene cluster (Figure 13). The structure of *phn* gene cluster exhibited a clear synteny between the Baltic Sea *N. spumigena* UHCC 0039 and its counterpart *N. spumigena* CCY941(Voß et al., 2013) which are geographically and evolutionarily closely related strains (Laamanen et al., 2001; Lyra et al., 2005). This indicates possibility of phosphonate degradation by *N. spumigena* in the Baltic Sea. However, difference in genetic construction was found between the Baltic Sea *N. spumigena* and brazilian isolate *N. spumigena* CENA596

(Popin et al., 2016) although close relationship has been demonstrated recently between *N. spumigena* UHCC 0039, CCY9414 and CENA596 through phylogenomic analyses (Teikari et al., 2018b). PCR screening with *N. spumigena* specific primers further showed amplification of key genes of *phn* gene cluster, *phnD* and *phnJ,* in all investigated *N. spumigena* strains showing the conservation of the *phn* gene cluster throughout the species. Amplification was not observed for *phnD* and *phnJ* genes in *N. sphaerocarpa* and *Aphanizomenon flos-aquae,* which further clarified that the designed primers for *phnJ* and phnD genes were highly specific to *N. spumigena* and could be used for the study of expression of *phn* gene cluster in Pi limitation and presence of phosphonates in *N. spumigena*. *phnD and phnJ* genes were also amplified from DNA extracted from *Nodularia* sp. UHCC 0099, *Nodularia* sp. UHCC 0158. This implies that *phn* gene cluster might be common in species of genus *Nodularia* as genetic exchange via horizontal gene transfer has been demonstrated before in natural populations of the *Nodularia* strains in the Baltic Sea (Barker et al., 2000). Growth assay could be performed to test if these tested *Nodularia* strains could utilize phosphonates. Further, the PCR products could be purified, sequenced and analyzed by bioinformatics-based methods could to assess the specificity of designed primers. In all, the primers designed in this study could be used to differentiate *phn* gene cluster containing and lacking cyanobacterial strains. Future use of primers for amplification of *phn* gene cluster from environmental samples demands the validation of these primers in multiple cyanobacterial species to conclude presence/absence of *phn* gene cluster.

6.2 Phosphonates as sole phosphorus source

Here, growth experiment on the Baltic Sea strains *N. spumigena* UHCC 0039 and UHCC 0060 harboring *phn* gene cluster, displayed variable preferences for provided phosphonates sources MPn, EPn and 2APn. MPn was the most preferred alternative source for both *N. spumigena* UHCC 0039 and UHCC 0060 (Figure 18, A and B). The other two phosphonate sources were not as preferable as MPn. 2APn was favored more by *N. spumigena* UHCC 0060 and EPn by UHCC 0039. In addition, the anthropogenic phosphonate sources tested could not fulfill the phosphorus requirement in *N. spumigena* (data not shown). These results suggest that naturally produced phosphonates can be utilized by the Baltic Sea *N. spumigena* among which MPn is the most preferable form. MPn is suggested to be produced by a marine archaeon *Nitrosopumilus maritimus* via MPnS-dependent pathway. The MPn biosynthesis in ocean is suggested to produce significant amount of MPn (Metcalf et al., 2012). Thus,

MPn may be a potential source of phosphorus for cyanobacteria with C-P lyase activity including the Baltic Sea cyanobacteria. Previous studies have also shown *phn* gene cluster harboring cyanobacteria capable of degradation of wide variety of phosphonates 2APn, MPn and EPn and glyphosate as sole source of phosphorus. These include several marine (Su et al., 2003; Dyhrmann et al., 2006) and freshwater (Ivanikova et al., 2008) cyanobacteria. The presence of different amino acid composition in PhnD proteins has been found to affect the dissociation constants to different phosphonates. This in turn can restrict the usable phosphonates and cause variations in phosphonates utilization between species and even strains (Forlani et al., 2008). A study conducted in *E. coli* (Alicea et al., 2011) showed that the PhnD protein have been found to have high affinity for naturally occurring phosphonates such as 2APn, EPn, MPn and relatively lower affinity for synthetic phosphonates glyphosate. The presence of *phn* gene cluster is thus not enough alone to degrade phosphonates and physiological studies are needed together with genomics data to find the suitable phosphonates for cyanobacteria

N. sphaerocarpa UHCC 0038 grew only in presence of Pi (Figure 18, C). The non-toxic *N. sphaerocarpa* UHCC 0038 displayed poor growth in Pi despite being in the same culture environment as toxic *N. spumigena* strains. Phosphonates were not suitable alternative source of phosphorus for this strain.PCR screening showed absence of *phnJ* and *phnD* genes in *N. sphaerocarpa* UHCC 0038 (Figure 17). The lack of *phn* gene cluster might be the reason for inability of *N. spaherocarpa* UHCC 0038 to assimilate phosphonates. Thus, the presence of C-P lyase complex in the Baltic Sea *N. spumigena* strains CCY9414 and *N. spumigena* UHCC 0039 and UHCC 0060 (this study) may be one of the factors that influences their dominance compared to other cyanobacteria in the Pi limited environments in the Baltic Sea. Furthermore, abundance of *N. spumigena* in the open-sea regions of the Baltic Sea may be attributed to their capacity to degrade phosphonates because these regions are not as heavily loaded by Pi inputs as in coastal areas.

6.2.1 MPn metabolism and methane release

Studies have shown that MPn has potential to serve as a source of phosphorus and carbon for bacteria during aerobic conditions (Cook et al., 1978; Ternan et al., 1998). MPn has been used as a sole source of phosphorus in bacteria such as *E. coli* and *Pseudomonas* during aerobic growth (Cook et al., 1978; White and Metcalf, 2007). Bacteria capable of using phosphorus but not carbon moiety might incorporate phosphorus into biomolecules and release methane into the environment (Draught et al.,

1979; Yakovleva et al., 1998). In this study, methane release from MPn was observed in starved *N. spumigena* cultures which were maintained under constant illumination (lower compared to nature) and nutrient rich medium. Methane was not released from cells growing in Pi. This indicates that MPn may be used primarily to fulfill the phosphorus requirement rather than carbon demand in *N. spumigena* and further implies that *N. spumigena* possess C-P lyase activity. A study (Gomez-Garcia et al., 2011) suggested that MPn could be utilized by *phn gene* cluster possessing *Synechococcus* OS-B' in the dark as carbon source. However, in the conditions with light, this strain assimilated phosphorus and released some of the reduced carbon as methane. *phn* gene cluster lacking *Synechococcus* OS-A' neither assimilated MPn as source of carbon nor emitted methane in dark and light conditions (Gomez-Garcia et al., 2011). The C-P lyase activity of *Trichodesmium* has been also determined in both field populations and laboratory cultures based on MPn and EPn hydrolysis with liberation of methane and ethane respectively (Beversdorf et al., 2010). The results in this study thus suggests that *N. spumigena* may contribute to aerobic methane release mediated by C-P lyase pathway and may explain the ambiguous supersaturation of methane in the surface of water of the Baltic Sea (Bange et al., 1999; 1998). However, more study is required to unravel the methane paradox in the Baltic Sea as recently methane accumulation in upper water column of the Baltic Sea during summer was attributed to methanogenic Archaea and zooplankton (Schmale et al., 2018). Further, it was found out that no methane production occurred in the Baltic Sea when copepod *Temora longicornis* was fed with *N. spumigena* (Stawiarski et al., 2018) because this cyanobacterium was not a suitable food source due to their toxic nature, large size and low lipid levels (Eglite et al., 2018). Therefore, toxic *N. spumigena* capable of C-P lyase activity might degrade MPn under Pi stressed conditions and release methane in similar way as shown by Karl et al. (2008).

6.3 Development of RT-qPCR as a tool to determine molecular marker

Molecular methods such as conventional PCR and qPCR have been used in studies of cyanobacteria (Sivonen, 2008). Most of these methods use DNA as a target molecule (Sivonen, 2008). PCR and qPCR have been used before to assess the distribution and abundance of toxin producing cyanobacteria in the Baltic Sea (Rantala et al., 2006; Koskenniemi et al., 2007). RT-qPCR can be used to study expression of genes of interest to determine a genetic marker at specific biological conditions. It is a very

sensitive technique and has lower detection limit than conventional PCR (Smith and Osborn, 2009).

RT-qPCR protocol developed in this study was based on the PCR amplification of target genes (*pstS*, *phnD* and *phnJ*) and a housekeeping gene *gyrB,* which was used for relative quantification of gene expression. Relative quantification by $2^{-\Delta\Delta Ct}$ method is used assuming that the efficiencies of both target and housekeeping genes are close to 100% and does not differ by more than 5% (Livak and Schmittgen, 2001). In this study, *gyrB* displayed consistent pattern of expression independent of control Pi and phosphonate treatments (MPn and 2APn. In addition, the efficiency of RT-qPCR reactions for all genes *phnJ*, *phnD, pstS* and *gyrB* were almost similar (98-100%) (Appendix, 2B).

Indiscriminate binding of non-specific SYBR Green can also result in fluorescence emissions in no template control due to binding of dye to primer dimers. Therefore, primers were designed on highly conserved region of the genes from *N. spumigena* (Figure 14), and reverse transcription and PCR steps were separated (Bustin et al., 2009). In addition, after completion of qPCR, melting curve analysis for all primers were performed that showed single melting peak in all conditions indicating primers are specific to target gene. Thus, the obtained information could be optimized and used in future to develop cyanobacteria-specific PCR method. This would eventually help in detection of phosphonate degrading strains in the Baltic Sea.

6.4 Responses of *N. spumigena* to Pi limitation and presence of phosphonates

6.4.1 Alkaline phosphatase activity as an indicator of Pi limitation

APA has been widely used as a marker for activation of *pho* regulon and Pi limitation in the environment (Van Wambeke et al., 2002; Hoppe, 2003, Dyrhman and Ruttenberg. 2006) since APA increases with Pi scarcity and vice-versa. During Pi-limited summer blooms of cyanobacteria in the Baltic Sea (Nausch et al., 2004), cyanobacteria may fulfill their P requirement from internal sources of P such as the polyphosphate storage granules (Healey, 1973). However, cellular phosphorus is also usually low in the Baltic Sea cyanobacteria (Nausch et al., 2004). The enzymatic hydrolysis of dominant ester form of DOP by alkaline phosphatase is a possible source of phosphorus for the Baltic Sea cyanobacteria (Kononen and Nômman, 1992). This enzyme has been shown to increase the fitness of *N. spumigena* during Pi limitation in the Baltic Sea (Vahtera et al., 2007). In this study, APA peaked in both *N. spumigena* UHCC 0039 and UHCC

0060 in noPi medium indicating lack of inorganic phosphate (Figure 19, A and B). Additionally, there was noticeable APA in both *N. spumigena* strains in 2APn but APA was lower compared to noPi. This indicates that presence of 2APn does not inhibit APA totally indicating that presence of phosphonate does not repress APA. This also suggests that 2APn is not a suitable source of phosphorus for *N. spumigena* and therefore, APA was induced in 2APn to degrade available phosphoesters. In medium containing Pi, APA was almost undetectable. APA also remained decreased in MPn conditions in comparison with noPi conditions in both *N. spumigena* strains. This suggests that MPn fulfilled the phosphorus demand and increased APA was not needed.

On the other hand, in *N. sphaerocarpa* UHCC 0038, APA was higher in Pi containing medium followed noPi medium (Figure 19, C; note different scales). This indicates that APA can be observed even in presence of inorganic phosphorus in the medium although activity of enzyme may be low. This is in accordance with study by Jansson et al., (1988), which showed that APA in cyanobacteria may be induced by different factors such as cellular phosphorus content, DOP substrate availability and the ability of specific cyanobacteria to assimilate Pi from the environment. APA has been observed in both marine and freshwater environments (Duhamel et al., 2010) and the enzyme activity was not always inversely related to Pi or total dissolved phosphorus concentration (Jansson et al., 1988; Karl., 2014). Even in the southern Baltic Sea, APA and Pi load were not found to be correlated (Schaub, 2012, personal communication). Hence, results from this study indicate that APA varies according to *Nodularia* species and thus this enzyme activity is a strain specific feature. Therefore, APA is probably not the most suitable marker to determine status of phosphorus in the cyanobacterial blooms and thus more sensitive methods would be better.

6.4.2 Transcriptional profile of *N. spumigena* during Pi limitation

Aiming to find suitable markers for Pi limitation the transcription level of three genes belonging to *pho* regulon *phnD*, *phnJ* and *pstS* was investigated by RT-qPCR in noPi, MPn and 2APn in the Baltic Sea cyanobacteria *N. spumigena* UHCC 0060 and UHCC 0039 to Pi limitation (Figure 25).

RT-PCR has been applied in cultured marine *Synechococcus* sp. strain and freshwater *Synechococcus* sp. to determine expression of *phnD* gene in Pi starvation (Ilikchyan et al., 2009). *phnD* gene expression was induced in Pi limiting environment suggesting that *phn* gene cluster is under control of *pho* regulon (Ilikchyan et al., 2009). In this study, RT-qPCR showed high expression of *phnD* in growth medium 2APn and

noPi indicating that *phnD* gene was upregulated in Pi limitation. Thus, *phnD* gene could be used as a marker for Pi deficiency as phosphonate transporter genes are more common in both marine and freshwater cyanobacteria (Scalan et al., 2009). Nonetheless, its suitability as a genetic marker is limited as *phnD* genes are not found in all genomes of cyanobacteria (Villarreal-Chiu et al., 2012).

Surprisingly, the constant upregulation of less common C-P lyase gene *phnJ* (Scalan et al., 2009) was obtained only in Pi removed medium supplemented by MPn in both strains. This implies that the expression of *phnJ* gene was dependent on the bioavailability of suitable substrate such MPn in this study. *phnJ* gene is generally used as a genetic marker for C-P lyase activity, and it is found in different cyanobacteria (Dyhrman et al., 2006; Adams et al., 2008; Voß et al., 2013). However, this gene has not been found in marine picocyanobacteria such as *Synechococcus* and *Prochlorococcus* (Scalan et al., 2009) with an exception as *Trichodesmium.* This emphasizes that *phnJ* gene might be common in brackish or freshwater adapted cyanobacteria. Thus, results suggest that *phnJ* may be potential marker for studying the availability of phosphonates in brackish aquatic ecosystems such as Baltic Sea.

The expression of *pstS* was shown to increase under Pi scarcity in cyanobacterium *Synechocystis* sp. 6803 (Suzuki et al., 2004). However, based on the RT-qPCR results from this study (Figure 22), the expression level of *pstS* gene did not increase in all conditions (Pi, MPn and 2APn) in both studied *N. spumigena* UHCC 0039 and UHCC 0060. This was different from RT-qPCR study where *pstS* has been used as a marker for Pi depletion in *N. spumigena* UHCC 0039 (in article, *N. spumigena* AV1) to study expression of nodularin synthease gene cluster in Pi limitation (Jonassaon et al., 2008). The study showed that *pstS* gene was highly expressed after 9-13 days of starvation when Pi level was not detected in the growth medium (Jonasson et al., 2008). Due to inconsistency in expression of *pstS*, a usual marker for Pi scarcity seemed unreliable based on our study. Similar were the results obtained in the study of diazotrophic *Crocosphaera watsonii* by Pereira et al. (2016). They suggested that a single genetic marker was not sufficient to characterize phosphorus stress but a combination of *pstS* and the arsenite efflux protein *arsB* generated better results. Thus, application of RT-qPCR to study gene expression in the environmental samples would aid in the chemical analysis and determination of phosphonates in the Baltic Sea, considering the fact that direct chemical analysis of phosphonates in natural aquatic ecosystems have been shown to be highly challenging (Cade-Menun et al., 2005).

7. CONCLUSIONS AND FUTURE PERSPECTIVE

In this study, *phn* gene cluster was readily detectable using PCR in the Baltic Sea *Nodularia* strains. Using biochemical methods, it was found that phosphonate sources could be assimilated by the Baltic Sea *N. spumigena*. The presence of *phn* gene cluster might have provided this strain the capacity to utilize phosphonates as alternative phosphorus source. MPn, the simplest of all organophosphonates was the most preferable form of phosphorus for *N. spumigena.* Furthermore, in Pi limited environment, MPn appeared likely to provide phosphorus than carbon to *N. spumigena*. Thus, the use of carbon-moiety by *N. spumigena* might release greenhouse gas methane as a by-product.

Alkaline phosphatase activity was detected both in presence of phosphonates and Pi. Its application as a marker for Pi starvation may not reliable to determine phosphorus status in cyanobacterial blooms. RT-qPCR assay was successfully developed to unravel the transcription response of three genes belonging to *pho* regulon. In general, all the genes of *pho* regulon are expected to be induced by Pi scarcity but this was not found in this study. The expression of *pstS* gene was not elevated neither in noPi nor in medium supplemented by MPn and 2APn. Thus, the *pstS* alone may not always be a suitable genetic marker for Pi scarcity and another gene(s) should be combined to detect Pi limitation within cyanobacterial populations. *phnD* gene was upregulated both in 2APn and noPi making it suitable indicator for at least Pi limitation. *phnJ* gene on the other hand, displayed consistent expression but required a suitable substrate MPn, likely increasing the possibilities of using this gene as a genetic marker of phosphonate bioavailability.

Naturally produced phosphonates are favorable source of phosphorus for the Baltic Sea cyanobacteria however, the determination of phosphonates concentration by chemical analysis is challenging. The use of gene expression studies in environmental samples could therefore provide some reliable genetic marker to determine the phosphonate status in the Baltic Sea. The molecular method developed in this study should be tested for natural water samples. There is further need of testing these *N. spumigena* specific primers with other cyanobacterial strains in order to quantify the *phn* gene cluster containing strains from environment. For this, optimization of designed method is required for e.g. probe based qPCR might be better in analyzing multiple samples than SYBR-green based methods. Furthermore, a wider

range of genes belonging to *pho* regulon should be studied in detail to find suitable markers for Pi stress and bioavailability of phosphonates.

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9. APPENDICES

Appendix 1: Z8 medium

Stock solution Z8I		**Stock solution Z8II-P (without phosphorous)**	
$NaNO_3$	46.7 g	KCl	2.7 g
$Ca(NO_3)_2\ 4\ H_2O$	5.9 g	Na_2CO_3	2.1 g
$MgSO_4 \cdot 7H_2O$	2.5 g	H_2O	1 l
H_2O	1 l		

Stock solution Z8II		**Stock solution Z8III**	
$K_2HPO_4\ .\ 3H_2O$	4.1 g	Fe-solution:	
Na_2CO_3	2.1 g	$FeCl_3 6H_2O$	2.8 g
H_2O	1 l	0.1 N HCl	100 ml

EDTA-solution		**Stock solution Z8Salt**	
(EDTA = Ethylene diamine tetra-acetate $Na_2\ 2H_2O$)		NaCl	87.5 g
EDTA	3.9 g	$MgSO_4 7\ H_2O$	37.5 g
0.1 N NaOH	100 ml	H_2O	1 l

Mix 10 ml of Fe-solution in 900 ml of deionized water.
Add 9.5 ml of EDTA-solution and add to 1000 ml.

Trace elements solution
Prepare the following stocks:

$Na_2WO_4\ 2H_2O$	0.330 g/100 ml	KBr	1.200g/100 ml
$(NH_4)_6Mo_7O_{24}\cdot 2H_2O$	0.880 g/100 ml	KI	0.830 g/100 ml
$ZnSO_4\ 7H_2O$	2.870 g/100 ml	$CuSO_4\ 5H_2O$	1.250 g/100 ml
$Cd(NO_3)_2\ 4H_2O$	1.550g/100 ml	V_2O_5	0.089 g/l
$Co(NO_3)_2\ 6H_2O$	1.460 g/100 ml	H_3BO_3	31.0 g
$(NH_4)_2Ni(SO_4)\ 6H_2O$	1.980 g/100 ml	$MnSO_4\ 4H_2O$	22.3 g
$Al_2(SO_4)_3K_2SO_4\ 24H_2O$	4.740 g/100 ml	$Cr(NO_3)_3\ 9H_2O$	0.410 g/100 ml

Prepare the solution by mixing 1 ml of solutions 1 to 10 and 12 plus 10 ml of solutions 11 and 13 in 700 ml in deionised water. Add to 1000ml. Store the solution in a brown glass

Z8-, Z8x- and Z8x-P-media		**Z8 SALT- and Z8xSALT-media**	
10 ml	Z8I or Z8IX-solution	100 ml	Z8salt-solution
10 ml	Z8II or Z8II-P-solution	10 ml	Z8I or Z8IX-solution
10 ml	Z8III-solution	10 ml	Z8II or Z8II-P-solution
1 ml	Trace elements solution	10 ml	Z8III-solution
		1 ml	Trace elements solution

Sparge 500 ml of water with CO2 for 20 minutes. Mix the above solutions in it and add to 1000ml with ordinary deionized water.Autoclave at 121 ° C for 15 minutes. After sterilization the pH should be 6.5-7.7.

Different Z8-variants in agarose
Prepare the desired Z8 variant by autoclaving the minerals and the agarose in separate bottles.
For example:In one bottle

10 ml	Z8I
10 ml	Z8II
10 ml	Z8III-solution
1 ml	Trace elements solution
470 ml	CO2 water

In another bottle (use a bottle which holds 1000ml)
5, 5 g Agarose (Invitrogen cat no 15510-019) in 500 ml deionized water
Autoclave at 121oC for 15 minutes.Cool the bottles to 42° C and combine the solutions and pour the plates.

Source: Kótai, J. (1972). Instructions for preparation of modified nutrient solution Z8 for algae. Norwegian Institute for Water Research, publication B-11/69. Bildern, Oslo.

Appendix 2

A. Alkaline phosphatase activity: preparation of standard and substrate

For preparation of MUF standard, 0.04 g of MUF (Sigma) was weighed and dissolved in 9 mL 20% CelluSolve (2-methoxyethanol) (Sigma Aldrich, lot #SHBF9695V) and 36 mL sterile water was added to make final volume 45 mL. Then the small aliquots with 500 µL were prepared and stored at -20 ºC. Similarly, 0.01 g of substrate 4-MUD (Sigma, lot# SLBN1289V) was dissolved in 1.2 mL of CelluSolve and 4.8 mL sterile water was added to make final volume 6 ml and stored at -20 ºC.

B.

Table 11 Efficiencies of primer pairs used during experimental RT-qPCR reactions

Target gene Annotation	Primer	Sequence 5'-3'	Product size	Efficiency %	R^2	Melting temperature (° C)
phnJ	phnJF	TTC TAG GGC GTG CAT TTT GC	216 bp	99.6	0.99	58
C-P bond lyase	phnJR	ACC AAC GCC GTG AAT ATT CG				58
phnD	phnDF	GGTGCCTGCGGATTCTGACA	225 bp	98.8	0.99	63
Phosphonate transporter	phnDR	TAACATCGCCGCGTCATGAG				60
pstS	pstSF	GTT GCA GCC AAT GGC ACT	119 bp	99.3	0.99	56
Phosphate binding	pstSR	CT TGAC TTG TGC CAA ACC				54
gyrB	gyrBF	CGC ATA TTC GCA CAC TGT TG	189 bp	100.5	0.99	58
Gyrase subunit B	gyrBR	TGT TGT AGT TGG CGT TGC TG				58